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An investigation of the nutritional, cultural, and biochemical factors which affect the growth and development of ericaceous plants.

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AN INVESTIGATION OF THE NUTRITIONAL, CULTURAL, AND BIOCHEMICAL FACTORS WHICH AFFECT THE GROWTH AND DEVELOPMENT OF ERICACEOUS PLANTS

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AN INVESTIGATION OF THE NUTRITIONAL, CULTURAL, AND BIOCHEMICAL FACTORS WHICH AFFECT THE GROWTH AND DEVELOPMENT OF ERICACEOUS PLANTS

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DEDICATION

To my wife and daughter who have changed my life style in innumerable ways.

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TABLE OF CONTENTS

TABLE OF CONTENTS (continued)

LIST OF TABLES

LIST OF FIGURES

INTRODUCTION

The nutritional, cultural, and biochemical aspects of the growth and development of ericaceous plants are an area of controversy. The most suitable nitrogen source is ammoniacal in origin according to certain investigators (1, 2, 8, 19, 29, 40, 46, 50, 82, 91, 93, 94) while others (6, 15, 16, 32, 68, 69, 70, 84, 90, 98) have reported that nitrate nitrogen (NO_3-N) and other carriers are as good as or better than ammonium nitrogen (NH_4-N) under certain conditions.

The actual nitrogen requirement is quite low when compared to other plants (91) and in fact high nutrient levels can prove deleterious to their growth (91).

Culturally, the ericaceous plants are naturally adapted to an acidic, organic medium, abundantly supplied with moisture, yet well aerated. The most successful pH range for ericaceous growth has been suggested as pH 4 to 5, (10, 23, 25, 45, 94, 97, 99). On the other hand, excellent growth has been obtained from 5 to 6.5 provided other nutritional factors were present in sufficient quantities (14, 46, 48, 63, 68, 70, 101).

Considerable work has been undertaken with iron and its relation to chlorosis in ericaceous plants. Many investigators (7, 23, 24, 48, 59, 63, 68, 69, 70) have implicated iron inactivation with chlorosis in Ericaceae under high pH conditions and attributed chlorosis under any conditions to iron inactivation.

The mycorrhizal associations with the Ericaceae add an interesting dimension to analyzing their growth and development as influenced by nutritional and cultural factors. Fungi are intimately associated with the roots and in certain cases the stems and leaves of the Ericaceae (2, 44, 52, 67, 75, 97). They have been implicated in nitrogen fixation, nitrate reduction and mineralization, and it has been difficult to interpret their effect upon the growth and development of ericaceous plants.

Cain (23) suggested that NO_3-N added to ericaceous plants on soils with low pH is readily reduced to ammonia by bacteria or to gaseous oxides of N and nitrogen gas by denitrifying bacteria. According to Alexander (3) the bacteria which bring about both types of reduction are extremely sensitive to pH, and acidic soils contain a sparse population of these bacteria, probably not large enough to account for economic losses of N. The population of bacteria becomes large only above pH 5.5. Thus, it seems difficult to believe that N applied as nitrate cannot be utilized by the Ericaceae since there are numerous reports of excellent growth with NO_3-N at low pH ranges. Biochemically, there has been very little work accomplished with the Ericaceae (40, 77, 92, 95). Oertli (70) advanced an explanation for the preferential use of NH_4-N over NO_3-N by ericaceous plants. He suggested that the absence of a biochemical system to utilize nitrates could be related to the restriction of blueberries to acid soils where NH_4-N is supposedly abundant and NO_3-N present in low concentrations. Townsend (91, 93, 94) suggested that NH_4-N was mandatory for the growth of the highbush and lowbush blueberries (Vaccinium corymbosum L. and

Vaccinium angustifolium Ait.) while Greidanus (40) indicated the necessity of NH_4-N for the growth of Vaccinium macrocarpon Ait. 'Stevens' (Stevens American Cranberry). He was unable to detect nitrate reductase activity in the leaves of these plants; however, activity was demonstrated in the roots.

This study was designed to interpret the effect of N source, pH, and a nitrification inhibitor on the growth and development of several ericaceous plants. An investigation of the nitrate reductase system was undertaken to provide evidence that NO_3-N can be assimilated by these plants.

LITERATURE REVIEW

Nitrogen Requirement of Ericaceous Plants

The literature concerning the growth and development of ericaceous plants is predominantly restricted to the highbush and lowbush blueberries, Vaccinium corymbosum L. and Vaccinium angustifolium Ait., the American cranberry, Vaccinium macrocarpon Ait. and several rhododendron species and cultivars, Rhododendron obtusum japonicum Planch, Rhododendron obtusum Planch 'Hexe', and Rhododendron ponticum L. Information concerning the woody, ornamental Ericaceae is lacking, but a basic understanding of nutritional and cultural factors which might affect the growth and development of these plants can be ascertained by studying the literature concerning the blueberry, cranberry, and rhododendron.

Most of the nutritional work on ericaceous plants has been descriptive in nature, that is, characterizations of the growth response due to different nutritional treatments. Various investigators have tried to correlate ericaceous plant growth in terms of pH (8, 14, 21, 24, 27, 43, 45, 46, 48, 63, 70, 86, 94, 95, 96, 97, 99), water table (46), media (10, 23, 30, 32, 43, 58, 82, 86, 97), nitrogen source and concentration (1, 2, 9, 15, 16, 23, 24, 29, 32, 40, 46, 50, 53, 58) 61, 69, 70, 82, 84, 86, 90, 91, 93, 94, 95, 98, 100), iron nutrition (7, 21, 24, 27, 32, 58, 63, 68, 69, 70, 100), phosphorus interactions (15, 48, 58, 63, 90, 100), all other macro and micro elements (8, 9, 21, 27, 32, 35, 58, 63, 70, 90,

100), mycorrhizal association (1, 2, 44, 52, 75, 97), nitrification (23), and enzymatic systems (40, 70, 77, 92, 95). In spite of the numerous studies, no one has formulated satisfactory conclusions concerning the growth and development of the Ericaceae as will be exemplified by the differing opinions concerning the previously mentioned areas of study.

Obviously, there is a complex meshwork of events which contribute to the maximum growth of ericaceous plants. Thusfar, no one has postulated a conclusive nutritional and cultural regime for these plants, probably because of the wide diversity of factors which affect their growth. A review of the nutritional and cultural requirements of several important cultivated Ericaceae follows.

Nutritional and Cultural Requirements of the Blueberry

Coville (30) in 1910 was the first to recognize that blueberries need an acidic, peaty soil, abundantly supplied with moisture, but also well aerated. He performed much of the early work on the domestication of the wild blueberry. Nutritional research work with the blueberries was not initiated until the 1930's. Doehlert and Shive (32) in 1936 were among the first investigators to look at the fundamental nutritional requirements of the highbush blueberry (Vaccinium corymbosum Ait. cv. Rubel) in greenhouse as well as field studies. They reported the highest yielding group of plants were those grown on high proportions of $Ca(NO_3)_2$ and low proportions of $(NH_4)_2SO_4$ in both sand and soil cultures. They concluded that the superiority of NO_3-N over NH_4-N was not surprising since the pH of the media was 4.5. They noted that blueberry has a low magnesium requirement along with a low but definite boron and manganese requirement.

Bailey and Everson (7) in 1937 sampled soil around chlorotic and healthy highbush blueberries and found that the pH was 5.2 in each case but that the iron content, as both the ferric and ferrous forms, was two- to fourfold greater under healthy plants. In greenhouse experiments, chlorosis was induced in plants by the addition of lime ($CaCO_3$). Chlorosis was evident on all limed soils, the severity increasing with the amount of lime added. Crystals of ferric citrate placed in a slit of a stem of a chlorotic plant caused the plant to turn green above and below the point of injection. They concluded that chlorosis in the blueberry plant was due to the lack of iron.

Chandler (27) in 1939 applied lime at the rate of 1, 2, 3 and 6 tons per acre to establish lowbush blueberry plants and increase their yield. The top 6 inches of the soil at the 6 ton per acre rate of lime application underwent a pH change from 5.0 to 6.6 and gave a significant yield increase over the plot receiving no lime.

Stene (86) in 1939 concluded that pH was not the controlling factor in the growth of the highbush blueberry cultured on NO_3-N since he found greater vegetative growth at pH 7.0 than at pH 4.0 or 5.2. He postulated that if there was an adequate source of nutrients in the root zone, pH played no role in regulating growth and development. He noted that nitrogen was an extremely important element in the maintenance of healthy, vigorous growth and also that organic matter contributed to increased blueberry growth.

Bailey (8) in 1941 studied lime and its effects on the growth of the 'Rubel' blueberry. He determined that lime limited or stunted its growth, for all plants receiving lime exhibited chlorosis, and the degree of chlorosis increased with increasing lime concentration. Lime at the rate of 9,408 pounds per acre produced a soil of pH 6.5 and reduced growth relative to soils receiving no lime at pH 4.6. This trend was apparent in a fine sandy loam and in the same soil amended with 5% (w/w) German peat.

Kramer and Schrader (58) in 1942 performed an extensive study into the nutritional requirements of the 'Cabot' highbush blueberry. The plants were grown in sand culture, and by proper manipulation of the mineral elements, they were able to produce deficiency symptoms for nitrogen, potassium, sulfur, calcium, boron, magnesium, phosphorus, and iron. They concluded that nitrogen is the limiting element in the normal growth of the blueberry plants.

Harmer (45) in 1945 defined the acidity range for satisfactory growth of the blueberry as between pH 4.0 and 5.2, with an optimum from pH 4.5 to 4.8. Kramer and Schrader (38) in 1945, working with the 'Cabot' highbush blueberry, tried to correlate the pH of the leaf sap with the growth and development of the blueberry. They determined that the leaf areas which were chlorotic were least acidic, the pH of such areas reaching as high as 5.0. The healthy leaves had a pH of approximately 3.5.

Cain (23) in 1952 was the first to put all the evidence together concerning blueberry nutrition. His work contradicted much of the early work concerning source of nitrogen, pH and other nutritional factors as they affect the growth

and development of the blueberry. He found that NH_4-N was superior to NO_3-N and that NO_3-N could prove injurious to the highbush blueberry. His experiments were conducted in a 1 : 1 peat:sawdust mixture, and pH was modified by appropriate methods. Blueberries were cultured at pH 5.2 and 7.0, and NH_4-N was superior to NO_3-N in facilitating growth at both pH's. There was no difference in response if the NH_4-N was added as hydroxide, chloride, sulfate, phosphate or nitrate; for growth was always superior to NO_3-N added as calcium, potassium, or sodium nitrate or as nitric acid. He noted that iron nutrition could be associated with NH_4-N in a metabolic manner. Plants receiving NO_3-N were chlorotic, but they contained as much or more iron in their foliage as the non-chlorotic plants. He postulated that the iron was rendered insoluble in the leaf tissue and unavailable for metabolism. He suggested that soil pH may not be a factor in iron absorption but more possibly affects microbial activity. Soils of high pH favor the development of nitrifying organisms and consequently a high concentration of nitrates, whereas acidic soils favor the development of denitrifying organisms and the conversion of nitrogen into the ammoniacal form.

Cain (24) in 1954 followed up the above work with a study of blueberry chlorosis as related to leaf pH and foliar composition. He noted that if the soil was above pH 5.0, chlorosis usually developed on blueberry plants unless nitrogen was maintained in the ammoniacal form. He postulated that Ca^{++}, Mg^{++}, and K^{+}, if in high concentrations in the medium, may be absorbed and result in increased leaf pH and precipitation of iron. Chlorotic leaves injected

with ammonium salts became green again. He mentioned that the symptoms produced by accumulated basic cations, iron deficiency, and those commonly occurring on calcarous soils are visibly identical. He suggested that the chlorosis may have resulted from the very low solubility of the ferric ion at the higher pH's. He also postulated the possibility of a specific low pH optimum for the enzymatic mechanism involving iron and that an enzymatic system involving iron could be inactivated by the various causes of chlorosis, i.e., lack of iron, high tissue pH, and the presence of certain heavy metals.

Cain and Galletta (25) in 1954 suggested that the optimum pH for the growth of the highbush blueberry was in the range of 4.5 to 4.8. This agrees with Harmer's (45) optimum pH range for blueberry growth.

Holmes (48) in 1960 studied the effects of pH and phosphorus on the chlorosis of the 'Roncocas' highbush blueberry in solution culture, and concluded that optimum growth depended on a proper balance of nutrients. The plants were grown at pH 4, 5, 6, 7 and 8 with five phosphorus levels. The greatest growth occurred at pH 4 and 5 while the higher pH levels resulted in chlorosis. Phosphorus reacted with iron, for the plants cultured on the low phosphorus level at pH 4 exhibited phosphorus deficiency and were only three-fourths as large as those grown on higher phosphorus treatments, whereas the plants grown in the highest phosphorus culture showed signs of iron chlorosis. If Fe-ethylenediaminedi-hydroxylacetic acid (Fe-EDDHA) was substituted for $FeCl_3$ in the solutions, the chlorotic plants became green, and all plants grew, flowered, and fruited. He also reported that chlorotic plants contained higher concentrations of iron in the leaf tissue than did the non-chlorotic plants.

Oertli (70) in 1963 showed that the source of nitrogen made little difference in promoting the growth of the 'Bluecrop' highbush blueberry. In nutrient culture NO_3-N produced growth of a similar magnitude to NH_4-N cultured plants at pH 4 and 6, provided iron was supplied as the chelate at the higher pH. When iron was supplied as $FeSO_4$, NH_4-N resulted in much greater growth. At pH 8 the plants were markedly reduced in growth regardless of iron source. The plants remained chlorotic but did not die in the course of the experiment. He disagreed with Cain's (23) previous work on blueberries and stated that NH_4-N or NO_3-N could suffice in the growth of these plants providing other factors are present.

Hall (43) in 1964 utilized four different media and a pH range from 4 to 7 for lowbush blueberry culture. He reported that best growth occurred at pH 4 to 5 and that the pH effect was more striking in some media than others.

Bradley and Smittle (17) in 1965 found that the most important factor affecting the growth of the 'Earliblue' highbush blueberry was the addition or omission of acid peat with the soil at planting. The acid peat produced the greatest growth. The use of ammonium sulfate, urea, ammonium phosphate, and sodium nitrate produced no significant growth differences. Chelated iron as Fe-EDDHA produced significant differences in growth when compared to the minus iron treatment.

Townsend (91) in 1966 reported that NO_3-N produced chlorotic conditions in the lowbush blueberry at a concentration of 14 ppm when grown in sand at pH 4.9, whereas NH_4-N under the same conditions resulted in excellent growth.

Nitrate N at 140 ppm proved lethal to the plant within three weeks of the initiation of the experiments, whereas plants under NH_4-N nutrition grew vigorously for three months when the experiment was terminated.

Cain and Eck (26) in 1966 extensively reviewed the literature concerning blueberry and cranberry culture and nutrition. They discussed history, species distribution and climatic requirements, growth and fruiting habits, soil requirements, pH requirements, nutrient requirements, foliar analysis, and fertilizer practices and recommendations.

Townsend (93) in 1967 noted that the 'Berkley' highbush blueberry grew better on NH_4-N or NH_4-N plus NO_3-N than on NO_3-N alone. Growth was significantly greater with NH_4-N nutrition than with the combined N sources. All work was performed in sand culture at a pH of 4.5.

Bailey (9) in 1966 found that the addition of ammonium sulfate to established 'Rubel' highbush blueberry plantings reduced fruit yield at the highest application rate of two pounds of $(NH_4)_2SO_4$ per bush. Herath and Eaton (46) in 1969 cultured 'Bluecrop' blueberries in raw Canadian peat and reported the greatest growth with NH_4-N. Their results suggested that NO_3-N was detrimental to the plants grown at pH 6 while NH_4-N had no deleterious effects at any of the four pH's (3.4, 4.3, 5.2, and 6.0).

Townsend (94) in 1969 found that pH and form of nitrogen affected lowbush blueberry growth and the nutrient levels in the leaves and roots. Plant growth in water culture on NH_4-N nutrition at pH 4.5 was superior to that of all other treatments. Poorest growth was obtained with NO_3-N at pH 6.0. He found

that NH_4-N at pH 6.0 or NO_3-N at pH 4.5 produced similar growth because of the compensating effects of favorable and unfavorable growth factors applicable to both.

Facteau and Eck (35) cultured 'Collins' highbush blueberry in a bentonite-sand substrate and found that the plants grew well within a wide pH range as long as essential elements were adequately supplied. Their results further suggested that high substrate cation levels could be detrimental to blueberry growth.

Trevett (97, 99) in 1970 in a series of papers recommended that for successful lowbush blueberry culture, both nitrogen and the pH are the essential factors regulating growth. He pointed out that in order to properly raise the blueberry the soil should be maintained around pH 4.5 to 5.0. Trevett and Durgin (98) reported that in unplowed podzolic soils with pH values no higher than 5.2, NH_4NO_3 was as effective and efficient as a source of nitrogen for lowbush blueberries as other standard carriers including $(NH_4)_2SO_4$, urea, and ureaform.

Johnston (50) in 1969 recommended the use of $(NH_4)_2SO_4$ for fertilizing blueberries if the pH is above 5.0 and urea if the soil pH is below 5.0. He advised avoiding nitrate and chloride fertilizers since they are sometimes toxic to the highbush blueberry.

Townsend (96) in 1971 reported that the growth of the 'Blueray' highbush blueberry was excellent within a pH range of 3.5 to 6.0. Growth was prevented at pH 2.5 and severely restricted at pH 3.0. He also indicated that growth was not limited by the direct effects of pH but possibly the effect of pH on ion availability.

Nutritional and Cultural Requirements of Cranberry

The cranberry as the blueberry is adapted to acidic and organic conditions, and its nutritional requirements are similar to those of the blueberry (1, 2). Beckwith (15, 16) reported that $NaNO_3$ resulted in a better crop of cranberries than those fertilized with a mixture of mineral and organic N sources. Relatively low amounts of N, 20 and 30 pounds per acre, gave adequate vine growth, but 30 pounds produced a greater fruit yield. He also noted that many of the early investigators recommended not applying $(NH_4)_2SO_4$ as it proved toxic to the cranberry. Addoms and Mounce (2) in 1932 reported that the 'Early Black' cranberry was capable of utilizing NH_4-N, NO_3-N, and glycine-N in both sand and soil culture. In sand culture NO_3-N was used under acidic but not alkaline conditions, while the opposite was true for ammonium. NH_4-N was readily absorbed and was detected in the tissues while NO_3-N was not detected in the cranberry or its mycorrhizae indicating that if NO_3-N is absorbed it was immediately reduced. Addoms and Mounce (1) in 1931 grew 'Early Black' cranberry in washed sand on $Ca(NO_3)_2$ and $(NH_4)_2SO_4$ at pH ranges from 4.9 to 5.6. The plants produced excellent runner growth on both NO_3-N and NH_4-N, exceeding that of similar plants grown in a bog. Again, NO_3-N was not detected in the cranberry plant at any time. Atwood and Zuckerman (6) in 1961 reported that yield responses from 'Early Black' cranberries growing in bogs were equally good using urea formaldehyde, $NaNO_3$, NH_4NO_3, $(NH_4)_2SO_4$, and urea, but that visual observations indicated less vine response on the plots treated with urea formaldehyde and $NaNO_3$. In terms of quality

factors urea formaldehyde and $NaNO_3$ resulted in no significant berry rot while NH_4NO_3, $(NH_4)_2SO_4$, and urea produced increasing percentages of rot in that order. Urea formaldehyde and $NaNO_3$ at rates of 80 pounds per acre did not affect the soundness of berries significantly, but the other three N carriers resulted in significant increases in rot even at the lowest rate of 20 pounds per acre. This indicated that N from a slowly available source and from NO_3-N was best suited for production of quality cranberry fruit. Kender and Childers (53) in 1959 studied the effect of urea formaldehyde, $Ca(NO_3)_2$, $(NH_4)_2SO_4$ and no fertilizer N on the growth of 'Early Black' cranberry using a typical bog soil in a controlled greenhouse experiment. They found that urea formaldehyde stimulated the greatest growth followed by NO_3-N, NH_4-N and no fertilizer.

Somogyi (82) in 1964 reported that urea and urea formaldehyde were most effective in promoting cranberry growth on a bog soil while $(NH_4)_2SO_4$ produced vigorous growth in both bog soil and a sand medium. The mixture of urea plus urea formaldehyde produced more growth than either separately. Torio and Eck (90) in 1969 reported that 'Early Black' cranberries grown in sand culture under NO_3-N for three seasons at 20 and 60 ppm N grew vigorously without chlorosis at a pH of 5.8. The higher N concentration produced the greater growth. Medappa and Dana (63) in 1970 grew cranberries in water culture with $Ca(NO_3)_2$ at pH values of 3, 4, 5, 6, 7 and 8. They found the greatest vegetative growth at pH 5 and 6. Plants grown at pH 6, 7 and 8 showed greater growth responses to Fe-EDTA than to $FeSO_4$; those grown at

pH 7 and 8 developed chlorosis when iron was supplied as $FeSO_4$. They concluded that chlorosis was not due to lack of iron but to inactivation of the metal in the tissue. Leschyson and Eaton (61) in 1970 grew 'McFarlen' cranberries in acidic peat (pH 3.4) on NO_3-N and urea-N. Nitrate N produced significantly greater vegetative growth than urea-N under these conditions. Greidanus (40) in 1971 reported that NH_4-N was essential for the growth of 'Stevens' cranberries in water culture, at pH 4.5, but that growth on NO_3-N was scanty. He concluded that cranberries have lost the genes necessary to synthesize nitrate reductase and consequently cannot grow on NO_3-N. His results do not agree with other investigators (15, 16, 61) who have reported NO_3-N to be as good as or better than an ammoniacal nitrogen source for cranberries.

Nutritional and Cultural Requirements of Rhododendron

The literature concerning this genus is limited and contradictory, similar to that of blueberries and cranberries. Spencer and Shive (84) reported that Rhododendron ponticum produced relatively good growth on 20 different nutrient solutions containing varying proportions of $Ca(NO_3)_2$ and $(NH_4)_2SO_4$ as N carriers. The medium was sand, and the pH was 4.0 in all cases. The combination of high NO_3-N and low NH_4-N resulted in chlorosis which was not overcome by the addition of supplemental iron. Barnette and Mowry (14) reported maximum growth of 'Formosa' azalea in soil culture fertilized with cottonseed meal and tankage at a soil pH range of 5.0 to 6.0. Plants grown below pH 5.0 made slow but healthy growth while those at or above pH 7.0 grew slowly and became

chlorotic. Ballhorn and Volz (10) reported that acidic sphagnum moss peat of pH 4.5 produced significantly better azalea plants than Iowa hypnam moss peat at pH 5.5. Foret and Volz (37) reported that a pH of 4.0 resulted in stunting and chlorosis of azalea. Colgrove and Roberts (29) reported that NH_4-N produced greater growth and better foliage color and was required in smaller amounts than NO_3-N for Rhododendron obtusum cv. Hexe grown in sand culture. The lower pH (3.5) produced greater growth with both the NH_4-N and NO_3-N sources. Plants grown on NH_4-N at pH 3.5 and 6.5 were superior to those cultured on NO_3-N at the same pH levels. They determined that chlorosis was closely associated with the pH of the leaf tissue. NH_4-N reduced pH, NO_3-N increased leaf pH and base absorption, and iron was inactivated as a result. Oertli (68) reported that $(NH_4)_2SO_4$, NH_4NO_3 and KNO_3 plus $Ca(NO_3)_2$ facilitated excellent growth of azalea in nutrient cultures supplied with chelated iron. Maximum vegetative growth occurred at pH 6.0 with all N sources, the poorest growth at 4.0, and intermediate growth at pH 8.0. The greater the N concentration in the nutrient solution the greater the decrease in growth. Full strength Hoagland's (47) to 1/3 Hoagland's (15 to 5 meq N, respectively) solution severely depressed growth while 1/8 to 1/20 Hoagland's (1.875 to 0.75) resulted in the greatest growth. In work of a similar nature Oertli (69) established that $(NH_4)_2SO_4$, NH_4NO_3, KNO_3 plus $Ca(NO_3)_2$ were adequate to produce vigorous, healthy azalea plants grown in nutrient culture of pH 6.0 provided iron as Fe-EDTA was added to the NO_3-N solution.

Biochemical Literature

The literature concerning the biochemistry of ericaceous plants is extremely limited. The explanations of the unique nutritional and cultural requirements of the Ericaceae have not been conclusively documented. The seemingly preferential use of ammoniacal nitrogen (23, 24, 29, 40, 46, 50, 82, 91, 93, 94, 95) by blueberries, cranberries, and rhododendrons suggests that these plants may be unable to reduce nitrate. If NO_3-N is absorbed by the plant, it must first be reduced by the nitrate reductase system to ammonia, from here the ammonia is incorporated into carbon skeletons and results in the formation of amino acids and ultimately proteins.

Several investigators (1, 40) failed to find NO_3-N in the tissues with which they were working and concluded NO_3-N was absorbed and reduced immediately following uptake. Others (1, 2, 44, 52, 65, 75, 97) have speculated that mycorrhizae play an important role in the growth and development of ericaceous plants. Mycorrhizae are symbiotic fungi which are able to form a sheath around plant roots and their hyphae penetrate the intercellular spaces as well as the intracellular areas. Addoms and Mounce (1) were unable to detect NO_3-N in the mycorrhizae. Addoms and Mounce (2) in 1932 reported that cranberry plants grown in sand culture were infected by the fungi from the root through the parenchymatous tissues of young stems, penetrating even the embryonic tip of the cranberry. Mycorrhizae (44, 52, 75) have been implicated in nitrogen fixation, nitrate reduction, and the mineralization of organic matter. It has been difficult to evaluate the effects of mycorrhizae on ericaceous plant growth, but ericaceous

plants grown in sterile culture have been able to grow without fungal infection (2, 97, 103).

Oertli (70) in 1963 was the first to suggest that the absence of a biochemical system to utilize NO_3-N would be an interesting explanation of the confinement of blueberries to acidic soils. He provided evidence that plants grown on NO_3-N would do as well as or better than those on NH_4-N provided other factors were present in optimum. Townsend (92) in 1966 was unable to demonstrate nitrate reductase activity in the leaves or roots of lowbush blueberry grown in a 1:2:1 peat:soil:sand mixture. He concluded that this plant does not possess the genetic complement necessary for the synthesis of a nitrate reducing system. However, Townsend (95) in 1970 detected nitrate reductase activity in the roots of lowbush blueberry grown on NO_3-N at pH 4 and 6 and on NH_4-N at pH 6.0. He was unable to detect activity in the leaves which are usually highest in nitrate reductase activity (78). Greidanus (40) in 1971 reported that cranberries did not respond to NO_3-N fertilization and that there was no nitrate reducing system in the leaves of these plants. He concluded that due to the environmental restrictions of the cranberry it has evolved without the genes necessary for the synthesis of nitrate reductase. Routley (77) in 1971 demonstrated nitrate reductase activity in the leaves of 21 of 24 ericaceous species and hybrids he examined. His work represented the first published account showing significant levels of nitrate reductase activity in the leaves of Ericaceae.

Conclusion

From the previous discussion it is obvious that no single source of nitrogen, pH range, and soil medium can absolutely and unequivocally be recommended for growing all ericaceous plants.

Ammonium N and NO_3-N, as well as urea, urea formaldehyde, organic sources and other nitrogen carriers have been reported as suitable N sources for the growth of various ericaceous plants.

Various investigators (14, 46, 48, 63, 68, 70, 96) have reported excellent growth of blueberry, cranberry, and rhododendron at pH ranges of 4 to 7. The general consensus seems to be that if iron is present in an available form the pH per se has little effect on growth. On the other hand, others (10, 23, 25, 45, 94, 97, 99) insisted that pH 4 to 5 was optimum for growing ericaceous plants.

The essential cation and anion requirements of the ericaceous plants are apparently low when compared to various agronomic and vegetable crops (91).

The growing medium best suited for ericaceous plant growth is described as being high in organic matter and well drained and aerated. Excellent growth of various ericaceous plants has also been reported in water culture (40, 48, 68, 69), sand culture (29, 32, 70, 84, 86, 90, 91), and in various soil mixtures (23, 35, 37, 43, 46).

The actual effect of mycorrhizae on the growth of ericaceous plants is open to question, for it has been demonstrated that ericaceous and other plants with and without the mycorrhizal associations grew equally well (19, 44, 52, 65, 97, 103).

The biochemical systems associated with nitrogen metabolism in the Ericaceae have received limited study (40, 77, 92, 95). Nitrate reductase activity was detected in the roots of the lowbush blueberry indicating that these plants were capable of utilizing NO_3-N. Routley (65) demonstrated nitrate reductase activity in the leaves of 21 species and cultivars of Ericaceae. Greidanus (40) demonstrated that cranberry roots, but not leaves, were capable of NO_3-N reduction and concluded that cranberry could be cultured only on NH_4-N.

Therefore, the aspects of nitrogen source, pH ranges and other cultural factors, and nitrogen utilization through specific biochemical systems must be critically studied in order to determine the optimum conditions for maximizing the growth and development of various woody ornamental ericaceous species.

MATERIALS AND METHODS

Plant Materials and Cultural Practices

The ericaceous plants chosen for study included bearberry (Arctostaphylos Uva-ursi L.), mountain laurel (Kalmia latifolia L.), drooping leucothoe (Leucothoe catesbaei Gray), sourwood (Oxydendrum arboreum L.), andromeda (Pieris japonica D. Don), Carolina rhododendron (Rhododendron carolinianum Rehder), Catawba rhododendron (Rhododendron catawbiense Michx.) cv. Roseum Elegans, and highbush blueberry (Vaccinium corymbosum L.). These species represent a broad cross section of the economically important ericaceous species.

Corn (Zea mays L.) cv. Harris Gold Cup, was employed in parts of this study because previous investigators (31, 41, 76) have indicated that the extraction and assays of nitrate reductase, malate dehydrogenase, and glutamate dehydrogenase from corn were relatively uncomplicated when compared to extraction of these and other enzymes from other plant species which are naturally high in inhibitory substances (4, 5, 49, 55, 57, 62, 73, 81, 89, 104, 105). All corn plants were grown in an unsterilized soil : peat : sand 7 : 3 : 2 mixture in flats and supplied with full strength Hoagland's (47) solution at every watering in quantities sufficient to completely wet the soil.

Unless specifically stated, all ericaceous plants were grown in an unsterilized 2 : 2 : 1 soil : peat : perlite mixture with a pH range of 4 to 5 and supplied with one-half strength Hoagland's solution at every watering in quantities

sufficient to completely wet the soil to insure adequate concentrations of NO_3-N in the growing medium. In addition to growing in soil, leucothoe and blueberry were grown in aerated solution culture on full strength Hoagland's solution at a pH of 6.5. This was done to secure root tissue which would be free of organic matter and soil particles and, therefore, suitable for enzymatic assays. Apple (Malus sylvestris Mill.), cv. McIntosh, was grown in sand culture on full strength Hoagland's solution applied at every watering. The leaf and root tissue was then utilized in various nitrate reductase studies.

All plant materials utilized for enzymatic determinations were greenhouse grown.

Greenhouse Experiments

Various nutritional and cultural experiments were conducted in the greenhouse with two-year old liners of leucothoe and Catawba rhododendron. The plants were grown in the unsterilized soil : peat : perlite mixture in six-inch standard plastic pots. The treatments consisted of three N sources, urea, potassium and calcium nitrate, and ammonium sulfate, supplied daily at a concentration of 100 ppm N and 150 ml volume. The specific composition of each nutrient is listed in the chart on the following page.

Soil pH was maintained in the ranges 4 to 5 and 6 to 7. The original pH of the soil : peat : perlite mixture was approximately 4.2. The pH was adjusted to approximately 6.2 by the addition of 18 g of CaO per 1000 g of potting mixture.

Salt	Nitrate	Urea	Ammonium
		g/liter	
KNO_3	0.255		
$Ca(NO_3)_2 \cdot 4H_2O$	1.09		
Urea		0.225	
$(NH_4)_2SO_4$			0.50
$MgSO_4 \cdot 7H_2O$	1.23	1.23	1.23
KH_2PO_4	0.68	0.68	0.68
$CaCl_2 \cdot 2H_2O$		0.73	0.73
KCl		0.37	0.37
H_3BO_3	2.86	2.86	2.86
$MnCl_2 \cdot 4H_2O$	1.81	1.81	1.81
$ZnSO_4 \cdot 7H_2O$	0.22	0.22	0.22
$CuSO_4 \cdot 5H_2O$	0.08	0.08	0.08
$H_2MoO_4 \cdot H_2O$	0.02	0.02	0.02
NaFeEDTA	0.0166	0.0166	0.0166

The nitrification inhibitor, 2-chloro-6-(trichloromethyl) pyridine[1] was applied as a soil drench at the initiation of the experiments and every two weeks thereafter at concentrations of 0 and 40 ppm.

[1] Trade name N-Serve, Dow Chemical Co., Midland, Michigan.

The experiment was designed as a split, split, split plot and was conducted in February and March of 1971 with eight single plant replicates for leucothoe and seven for Catawba rhododendron. The experiment was repeated during August and September of 1971 using four replicates for each species.

In the February-March experiments the effects of the various treatments on growth were of principal concern. Growth was evaluated on the basis of total length of shoots, total number of shoots, and the overall vigor and appearance of the plants.

In the August-September experiment growth was again evaluated, but measurements of final soil pH, NO_3-N content of the soil and the young and old leaf tissue, and NH_4-N concentrations in the soil were determined. Leucothoe was the only species evaluated on all of the above parameters, as rhododendron did not respond to the various treatments, probably due to the high greenhouse temperatures and the onset of dormancy. Leaf samples were harvested at this time, rinsed in deionized water, dried and treated as described under Nitrate Determinations of Leaf and Soil Samples. All soil samples were collected after the six-week experimental period and immediately frozen until the analyses were conducted.

Determination of pH

Fifty grams of soil at field capacity were suspended in 100 ml of distilled water and periodically stirred for 15 minutes. The suspensions were then decanted through two layers of "Kim-Wipe" tissue, and the pH of the filtrate was determined with a Beckman Expandomatic pH meter.

Nitrate Determination of Leaf and Soil Samples

Two hundred to 400 mg samples of dried leaf tissue which had been ground in a Wiley mill and which had passed through a 20-mesh screen were placed in 125 ml Erlenmeyer flasks. Fifty ml of distilled water were then added to the flasks. They were agitated on a Burrell Wrist-Action Shaker for 15 minutes. The suspension was then filtered through glass wool, and the NO_3-N concentration of the extract was determined with an Orion Research Ionalyzer, Specific Ion Meter, Model 401.

Soil NO_3-N determinations were accomplished by suspending 50 g of soil at field capacity in 100 ml of distilled water and periodically stirring the suspension for 15 minutes. The suspension was then filtered through "Kim-Wipe" tissue, and NO_3-N was determined as outlined above.

Soil Ammonium Nitrogen Determinations

As previously described, 50 g of soil at field capacity were mixed with 100 ml of water. The resultant suspension was then filtered through Whatman No.1 filter paper in a Buchner funnel into a 250 ml side-arm Erlenmeyer flask. The entire sample was then transferred to the holding flask of a steam distillation apparatus. The sample was made alkaline by the addition of 5 to 10 ml of 40% NaOH, and distillation was carried out at 90° C for 30 minutes. A 2% boric acid solution containing 10 ml/liter of an indicator solution consisting of 0.2 g methyl red made to 100 ml with 95% ethanol and 1.2 g methylene blue made to 100 ml with 95% ethanol and mixed in the proportion 2: 1 (v/v) methyl red: methylene blue was used to trap the ammonia distilled from the soil extracts.

The ammonia present in the boric acid indicator solution was determined by titration with $^{N}/_{70}$ $KH(IO_3)_2$. Each ml of $KH(IO_3)_2$ used was equal to 0.2 mg of ammonium nitrogen.

Blueberry Greenhouse Experiment

Thirty-three-year old 'Jersey' highbush blueberry plants were grown in the unsterilized soil : peat : perlite mixture as previously described. Three N sources, ammonium sulfate, potassium nitrate plus ammonium sulfate, and potassium nitrate were supplied daily at a concentration of 50 ppm N in 250 ml volume. The essential elements were supplied at one-half Hoagland's (47) concentration. The two pH ranges were established as previously described under Greenhouse Experiments. The treatments were initiated on 21 June 1971, and leaves were sampled for nitrate reductase activity 30 and 50 days later. Roots were sampled for nitrate reductase activity on 11 August 1971.

Enzyme Extraction and Assay Techniques

Nitrate Reductase ($NADH_2$: nitrate oxidoreductase-EC 1.6.6.2)

Two methods were employed for extracting nitrate reductase from the leaves and roots of plants utilized in this study. The first was named the tissue homogenate extraction and assay technique, the second, the tissue infusion extraction and assay technique.

Tissue Homogenate. This method is essentially the same as that described by Hageman and Flesher (41). The leaf or root tissue was homogenized in a solution of 0.2 M tris, 0.01 M L-(+)-cysteine, and 3×10^{-4} M EDTA adjusted to pH 7.5

with 2 N HCl. The ratio of extraction medium to tissue was 4:1 (v/w). All grinding was accomplished with a Virtis '45' homogenizer at medium speed for one minute.

The homogenate was then filtered through four layers of cheesecloth and centrifuged for 15 minutes at 10,000 g in a refrigerated centrifuge at 0° C. The supernatant was then decanted and used for assays while the pellet was discarded. All operationswere carried out in an ice bath to prevent enzyme denaturation.

The assay mixture consisted of 1.5 ml 0.1 M potassium phosphate buffer pH 7.5, 0.2 ml of 0.1 M KNO_3, 0.5 ml of 1.36×10^{-3} M NADH, and 0.5 ml of enzyme extract for a final volume of 2.7 ml. The reaction was initiated by the addition of NADH. The reaction mixture was incubated in a water bath at 30° C for a prescribed time, and the reaction stopped by adding 1 ml of 1% w/v sulfanilamide in 1.5 N HCl. One ml of 0.02% w/v N-(1 naphthyl) ethylene-diamine dihydrochloride in 0.2 N HCl was added, and the color was allowed to develop for 5 minutes. The absorbancy was determined by reading each sample against its own blank (complete except for NADH) in a Beckman DU-2 spectro-photometer at 540 mm.

Tissue Infusion. This method is a modification of Mulder's (11, 66) procedure for nitrate reductase extraction and assay. Leaf or root tissue was sectioned into mm-sized pieces and placed in a 22 mm by 200 mm test tube. To the test tube were added 5 ml 0.1 M potassium phosphate buffer, pH 7.5, 1 ml of 0.1 M succinate neutralized to pH 7.0 with NaOH, 1 ml of 0.1 M KNO_3, and 2 ml

distilled water. This mixture was then incubated in a water bath at 30°C for a prescribed time. The reaction was stopped, and the color was developed by filtering the incubation solution directly into a 250 ml Erlenmeyer flask containing 2 ml of 1% w/v sulfanilamide in 1.5 N HCl plus 2 ml of 0.02% w/v N-(1-naphthyl)-ethylenediamine dihydrochloride in 0.2 N HCl. The color which developed with these reagents was read at 540 nm with a Beckman DU-2 spectrophotometer.

Glutamic Dehydrogenase (L-glutamate: NAD^+ oxidoreductase (deaminating), EC1.4.1.2)

Leaf tissue was washed, blotted and ground in a solution of 0.05 M tris, pH 7.5 and 0.4 M sucrose (4 ml of buffer per gram of tissue) for one minute with a Virtis '45' homogenizer. The homogenate was filtered through four layers of cheesecloth and centrifuged for 15 minutes at 10,000 g. The supernatant was then used for the assays, and the pellet was discarded.

Reductive Amination of α-Ketoglutarate. The assay described is similar to that of Pahlich and Joy (71). Enzyme extract (concentration dependent upon activity) was added to ± 2 ml 0.2 M tris, pH 8.0, 0.2 ml 0.2 M α-ketoglutarate, pH adjusted to 7.5 with NaOH, 0.2 ml 3.2 M $(NH_4)_2SO_4$ and 0.5 ml 1.36×10^{-3} M NADH for a final volume of 3 ml. The reaction was initiated by adding NADH, and the change in optical density was determined with a Beckman DU-2 spectrophotometer at 340 nm. The blank included everything except NADH. α-ketoglutarate was also excluded from the assay mixture to determine if NADH was oxidized by a system other than glutamate dehydrogenase.

Malic Dehydrogenase (L-malate: NAD oxidoreductase, EC 1.1.1.37)

The extraction of this enzyme was exactly as described for glutamate dehydrogenase.

Reduction of Oxaloacetate. The assay was similar to that described by Danner and Ting (31). Enzyme extract (concentration dependent upon activity) was added to ± 2 ml 0.2 M tris, pH 8.0, 0.2 ml 0.01 M oxaloacetate adjusted to pH 7.0 with NaOH, 0.5 ml 1.36×10^{-3} M NADH for a final volume of 3 ml. The reaction was initiated by adding NADH, and the change in optical density determined with a Beckman DU-2 spectrophotometer at 340 nm. The blank included everything except NADH. Oxaloacetate was excluded from the assay mixture to determine if NADH oxidation was affected by a system other than malic dehydrogenase.

Inhibitor Studies

Nitrate Reductase

Due to the inability to extract an active nitrate reductase from leaf and root tissue of the ericaceous plants sampled, as well as leaf and root tissue of apple, and roots of corn, various manipulations of the tissue homogenate were undertaken.

Several investigators (4, 39, 55, 57, 73, 81, 105) have extracted active enzyme preparations from plant tissue abnormally high in phenolic compounds.

Phenolics, more specifically tannins (49, 89, 105), have been been shown to be potent precipitators of protein. Workers (55, 62) have reasoned that if the phenolics could be complexed by some substance before they react with the

protein, then an active enzyme fraction could be extracted. Although many substances have been used (4, 5, 49, 55, 62, 73, 81, 105), the most successful has been polyvinylpyrrolidone (PVP).[2] PVP was used according to the directions of Klepper and Hageman (57) for extracting an active reductase fraction from the various plants.

Other experiments were conducted with PVP to determine if the compound did complex phenolic substances. The various manipulations are described in the appropriate area under Results.

Dialysis Experiments. Extracts of leucothoe and Catawba rhododendron prepared with and without PVP were dialyzed against a large volume of distilled water at 4°C for periods of 24 and 48 hours. The water was changed at least four times during the dialysis period. The dialysate was then utilized in nitrate reductase assays as previously described.

Characterization of the Inhibitory Substances in Leucothoe and Rhododendron

Various experiments were conducted to determine if the inhibitory substances were of a macro- or micromolecular nature. Since phenolic type compounds had been previously implicated in enzyme inactivation (104), other experiments were designed to explore the possibility of phenolic inactivation of protein in the two ericaceous species.

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[2] The insoluble form was used in this study, and it goes under the trade name Polyclar AT (GAF Corporation Dyestuff and Chemical Division, 140 West 51 Street, New York, N. Y. 10020).

Boiling. Extracts were boiled for 15 minutes, and after centrifugation at 5000 g for 5 minutes were added back at equal volumes (0.5 ml) to 0.5 ml active corn nitrate reductase extracts and the percent inhibition determined.

Dialysis. Extracts prepared with and without PVP were dialyzed as described previously. The dialysate was then added back to active corn extracts as previously described.

Inhibitor Stability. Extracts prepared without PVP were allowed to remain on the laboratory bench for seven days at the end of which time they were added back to active corn extracts as previously described.

Polyvinylpyrrolidone. Plant material from leucothoe, Catawba rhododendron, and other ericaceous species was homogenized with PVP. The supernatant was assayed for nitrate reductase activity and also added to active corn extracts. In several studies PVP was added to the original PVP prepared crude ericaceous extract in saturating quantities. The extract plus PVP was centrifuged at 10,000 g for 5 minutes, and the supernatant added to corn extracts as previously described.

Fractionation Study. This experiment was designed to determine if the inhibitor was of a macro- or micromolecular nature. The following flow diagram represents the steps used to separate the two fractions:

LEUCOTHOE OR RHODODENDRON

LEAF TISSUE

↓ Homogenized as described under Tissue Homogenate method.

SUPERNATANT

↓ Precipitated with 4 volumes of cold 80% acetone and centrifuged at 10,000 g.

SUPERNATANT ← → PELLET

SUPERNATANT

↓ Taken almost to dryness in an evaporator.

CONCENTRATE

↓ Dissolved in 0.1 M KPO_4 buffer, pH 7.5, using the same volume of buffer used in the homogenization.

↓ Centrifuged at 5,000 g for 15 minutes.

SUPERNATANT added back to active corn extracts in equal volumes (0.5 ml).

PELLET discarded

PELLET

↓ Washed in cold distilled water five times.

Resuspended in 0.1 M KPO_4 buffer, pH 7.5, using the same volume of buffer used in the homogenization.

↓ Centrifuged at 5,000 g for 15 minutes.

SUPERNATANT added back to active corn extracts in equal volumes (0.5 ml).

PELLET discarded

The resuspended concentrates and pellets were then added back to active corn extracts as previously described.

Spectral Studies. Andersen and Sowers (4) have indicated that qualitiative determinations of phenolic compounds in plant tissue were possible by measuring the absorbancy of various solutions in a range of wavelengths from 220 to 420 nm. Absorbancies of corn, blueberry, Catawba rhododendron, and corn extracts which had been treated in various manners as described under the appropriate figures, were determined over wavelengths from 250 to 360 nm. The relative absorbancies of these solutions were correlated with the percent reduction to active nitrate reductase fractions from corn.

Exogenously Applied Phenolic Compounds. Various phenolic compounds including p-dihydroxybenzene (hydroquinone), 3, 3', 4', 5, 7-pentahydroxy-flavone-3-rutinoside (rutin), 1, 2, 3 trihydroxybenzene (pyrogallol), 5-hydroxy-1, 4-napthquinone (juglone), 1, 3, 4, 5-tetrahydroxycyclohexanecarboxylic acid 3-(3,4)-dihydroxycinnamate (chlorogenic acid), 1, 3, 4, 5-tetrahydroxy-cyclohexanecarboxylic acid (quinic acid), 3, 3', 4', 5, 7-flavanpentol (catechin), 3, 3', 4', 5, 7-pentahydroxyflavone (quercitin), and Tannic acid were added to active corn extracts in equal volumes (0.5 ml). The concentration of all phenolic compounds was 10^{-3} M, and those which exhibited inhibitory properties were examined at lower concentrations.

Nitrate Reductase Induction Study

Recently, Routley (77) indicated that significant levels of nitrate reductase could be induced in the leaves of 21 of 24 ericaceous species. The tissue was incubated under high-NO_3-N and high-light intensity, both of which have been shown to influence nitrate reductase activity in various plants (17, 18, 33, 34, 36, 41, 42, 51, 56, 77, 87, 88, 102).

Leaf tissue sections approximately 10 mm by 20 mm of Leucothoe catesbaei, Pieris japonica, Rhododendron carolinianum and Rhododendron catawbiense were incubated in a 200 ml solution of 0.05 M PO_4 buffer, pH 7.5, containing chloramphenicol, 50 µg/ml, and NO_3-N, 500 µg/ml. Leaf tissue was also incubated in a similar medium but propanol was added at a concentration of 5% (v/v). Five-hundred-ml Erlenmeyer flasks, containing the tissue and solutions, were placed in an incubator at 25° C and continuously illuminated (500 ft-c). All solutions were aerated during the 24 hr incubation period. The tissue was washed five times with distilled water and assayed for nitrate reductase as described under the tissue infusion methodology. Nitrate content of the incubated tissue was determined as previously described and expressed as percent NO_3-N on a dry weight basis.

Chromatography Study

Concentrated extracts of leucothoe and Catawba rhododendron prepared with and without PVP were spotted along with known tannic acid and chlorogenic acid fractions. The chromatography paper used was Whatman 3mm, and prior to use the paper was run in distilled water for two days. The solvent system was a 4:1:5 butanol: acetic acid: water mixture, as this has been shown to be an excellent solvent system for phenolic compounds (20). Descending chromatography was employed in all cases. The chromatograms were dried and scanned under ultraviolet light for fluorescence properties. Ferric salts including 0.1% w/v $FeCl_3$ and 0.2% w/v $FeNH_4(SO_4)_2 \cdot 12 H_2O$ were sprayed on the chromatograms

and also added to the extracts. These salts result in the appearance of green or grey-blue spots, depending whether the tannin was composed of catechol or pyrogallol residues, respectively.

Bacterial and Mycorrhizal Study

Bacterial Experiments

Due to the fact that bacteria are able to reduce nitrate (54), it was necessary to establish whether bacteria were present on the leaf surfaces and also insure that no bacterial growth could occur during the incubation period. Potato dextrose[3] agar was used as the synthetic growth medium. The agar was prepared by suspending 39.0 g of dehydrated agar in 1000 ml of distilled water. The contents were mixed thoroughly and heated until boiling. The resultant solution was dispensed into Petri dishes and autoclaved at 121° C for 15 minutes.

Mature leaves of leucothoe were harvested and washed by placing in distilled water or 2 and 4% hypochlorite solutions for one minute. The leaf tissue was sectioned as described under the tissue infusion methodology. Two tissue sections were transferred to sterilized Petri dishes, and each treatment was replicated three times. The Petri dishes were placed in an incubator at 25° C for four days at the end of which time they were examined for bacterial growth and photographed.

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[3] Fisher Bio Cert, Fisher Scientific Company, Fair Lawn, New Jersey.

Leucothoe leaf tissue was washed as described previously and then assayed by the tissue infusion method for nitrate reductase activity.

Chloramphenicol (79) has been shown to be an effective inhibitor of protein synthesis in bacteria and fungi. Various investigators (41, 51, 56) utilized chloramphenicol in their incubation medium to insure that bacteria were not affecting nitrate reductase activity. Chloramphenicol (30 μg/ml) was shown to prevent bacterial growth without affecting the activity of nitrate reductase (79). Therefore, when the tissue infusion method was used, chloramphenicol (30-50 μg/ml) was added to the incubation mixtures.

Mycorrhizal Study

The presence or absence of mycorrhizae in various ericaceous roots was determined by the differential staining technique of Philips and Hayman (72). The following plants were greenhouse grown under high nutrient levels: leucothoe in soil and water culture, Catawba rhododendron in soil, and highbush blueberry in water culture. Roots were collected from these plants and also a wild highbush blueberry, washed free of organic matter and soil particles and placed in 125 ml Erlenmeyer flasks containing a 10% KOH solution, heated for one hour at 90°C, and washed in distilled water. They were then immersed for 10 minutes in a 3% H_2O_2 solution to clear them, rinsed thoroughly in distilled water to remove the H_2O_2, and acidified in 0.01 N HCl. They were then stained by simmering for 5 minutes in 0.05% trypan blue in lactophenol, and the excess stain removed in clear lactophenol. The stained roots were then

mounted on a glass slide with a cover slip and photographed with a specially adapted Bausch and Lomb photomicroscopy outfit. The magnifications are listed for each photomicrograph appearing in the results.

Statistical Methods

The data were analyzed using an analysis of variance, standard deviation, or Duncan's multiple range test as described by Steel and Torrie (85). Differences were tested at the 1% and 5% level of probability.

RESULTS

Greenhouse Experiments

February and March Leucothoe Study

The growth and development of leucothoe were significantly affected by the source of N. Plants grown on nitrate N had a significantly greater number and length of shoots when compared to those cultured on urea or NH_4-N (Table 1). Nitrate N resulted in approximately twice as much new shoot growth as urea and NH_4-N. Urea and NH_4-N treatments also resulted in tip and marginal necrosis which first appeared five weeks after the initiation of the treatments (Table 1). The necrosis first appeared on the older foliage and with continued application of urea and NH_4-N eventually occurred on the young leaves. Necrosis did not occur when NO_3-N was used (Figures 1 and 2).

The pH of the growing medium resulted in marked differences in growth (Table 2). Both the number and length of shoots were significantly greater at pH 6 to 7 than at pH 4 to 5. The overall appearance of leucothoe was not affected by the pH of the medium.

The presence or absence of the nitrification inhibitor had no significant effect on the growth or appearance of leucothoe (Table 3).

TABLE 1

EFFECT OF SOURCE OF NITROGEN ON THE GROWTH OF

LEUCOTHOE CATESBAEI

Source	GROWTH INDEX OF SHOOTS		
	Number	Length (in.)	Appearance [x]
$(NH_4)_2SO_4$	6.7 a [y]	26.5 a	2.8 a
Urea	7.5 a	26.0 a	3.1 a
KNO_3 and $Ca(NO_3)_2$	9.3 b	44.8 b	1.1 b

[x] 1 = 0-25% browning; 2 = 26-50%; 3 = 51-75%; 4 $\geq$ 76%.

[y] $P \leq 0.05$

FIGURE 1. EFFECT OF UREA AND NO_3-N UPON THE APPEARANCE OF LEUCOTHOE CATESBAEI.

FIGURE 2. EFFECT OF NH_4-N AND NO_3-N UPON THE APPEARANCE OF LEUCOTHOE CATESBAEI. THE OLD FOLIAGE IS MOST SEVERELY AFFECTED.

TABLE 2

EFFECT OF SOIL MIXTURE pH ON THE GROWTH OF LEUCOTHOE CATESBAEI

	GROWTH INDEX OF SHOOTS		
pH	Number	Length (in.)	Appearance x/
4	7.2*	25.5**	2.5 ns
6	8.5	35.4	2.2

x/ 1 = 0-25% browning; 2 = 26-50%; 3 = 51-75%; 4 _ 76%.

* Significant at P _ 0.05

** Significant at P _ 0.01

TABLE 3

EFFECT OF A NITRIFICATION INHIBITOR (2-chloro-6-trichloromethyl-pyridine) ON THE GROWTH OF LEUCOTHOE CATESBAEI

Treatment ppm	GROWTH INDEX OF SHOOTS		
	Number	Length (in.)	Appearance [x]
0	8.1 ns	33.6 ns	2.5 ns
40	7.6	31.3	2.2

[x] 1 = 0-25% browning; 2 = 26-50%; 3 = 51-75%; 4 ≥ 76%.

There were no significant interactions between or among N source, pH and nitrification inhibitor in any of the indices used to measure growth.

Rhododendron Study

This ericaceous species did not respond to the various treatments in the same degree as leucothoe. The source of N had no significant effect upon the number and length of shoots (Table 4), but the appearance of the plant was significantly better under NO_3-N nutrition (Figure 3 and Table 4). Urea and NH_4-N, as in the case of leucothoe,resulted in the tip and marginal necrosis of the old foliage. The degree of injury was not as severe in the case of rhododendron, but the necrosis, nonetheless, resulted in a very unsightly plant (Figure 3 and Table 4).

The pH of the growing medium had no significant effect upon growth or the appearance of the plants (Table 5). The fact that growth at pH 6 to 7 was as good as that at pH 4 to 5 is worthwhile noting in view of previous reports (29) concerning rhododendron growth and pH.

The nitrification inhibitor had no significant effect upon the growth or appearance of rhododendron (Table 6).

As in the leucothoe study, there were no significant interactions between or among N source, pH and nitrification inhibitor in any of the indices used to measure growth.

TABLE 4

EFFECT OF SOURCE OF NITROGEN ON THE GROWTH OF

RHODODENDRON CATAWBIENSE cv. ROSEUM ELEGANS

Source	GROWTH INDEX OF SHOOTS		
	Number	Length (in.)	Appearance [x]
$(NH_4)_2SO_4$	2.8 a [y]	4.8 a	1.5 a [z]
Urea	3.0 a	5.5 a	1.7 a
KNO_3 and $Ca(NO_3)_2$	2.4 a	5.2 a	1.0 b

[x] 1 = No browing; 2 = Tips browned; 3 = Lower leaves 50% browned.

[y] $P \_ 0.05$

[z] The data do not correspond with the appearance of NH_4-N and urea treated plants in Figure 3 as the picture was taken several weeks after the data had been collected.

FIGURE 3. EFFECT OF UREA, NO_3-N, AND NH_4-N ON THE APPEARANCE OF RHODODENDRON CATAWBIENSE cv. ROSEUM ELEGANS.

TABLE 5

EFFECT OF SOIL MIXTURE pH ON THE GROWTH OF

RHODODENDRON CATAWBIENSE cv. ROSEUM ELEGANS

pH	GROWTH INDEX OF SHOOTS		
	Number	Length (in.)	Appearance x/
4	2.7 ns	4.4 ns	1.43 ns
6	2.8	5.5	1.38

x/ 1 = No browning; 2 = Tips browned; 3 = Lower leaves 50% browned.

TABLE 6

EFFECT OF A NITRIFICATION INHIBITOR (2-chloro-6-trichloromethyl-pyridine) ON THE GROWTH OF RHODODENDRON CATAWBIENSE cv. ROSEUM ELEGANS

Treatment ppm	GROWTH INDEX OF SHOOTS		
	Number	Length (in.)	Appearance x/
0	2.6 ns	5.3 ns	1.40 ns
40	2.8	5.0	1.40

x/ 1 = No browning; 2 = Tips browned; 3 = Lower leaves 50% browned.

August and September Leucothoe Study

The source of N was responsible for some striking differences in the vegetative growth and appearance of leucothoe. Interestingly, both urea and NO_3-N produced excellent growth while NH_4-N was virtually ineffective in stimulating growth. Urea and NO_3-N resulted in 12.4 and 12.3 new shoots per plant and 60.7 and 59.6 inches of new vegetative growth per plant, respectively (Table 7). On the other hand, NH_4-N produced only 6.1 new shoots per plant and 26.1 inches of new growth (Table 7). The implication is that both urea and NO_3-N were vastly superior to NH_4-N which is contrary to the results obtained with other ericaceous plants (19, 40, 46, 50). Although a numerical evaluation of necrosis was not conducted in this experiment, it was observed that the toxicity symptoms first appeared after five weeks and that both urea and NH_4-N treated plants were severely injured while those on NO_3-N showed no injury. This would seem to eliminate urea as an N source even though the vegetative growth was as good as that produced with NO_3-N.

The pH of the growing medium had no significant effect upon growth as is evident from the data in Table 8. As in the February-March experiment, there were no chlorotic leaves on plants cultured at pH 6 to 7.

The presence or absence of the nitrification inhibitor had no significant effect upon growth (Table 9). From this and the previous studies, it seemed that the use of a nitrification inhibitor was of little or no benefit.

TABLE 7

EFFECT OF SOURCE OF NITROGEN ON THE GROWTH OF

LEUCOTHOE CATESBAEI

Source	GROWTH INDEX OF SHOOTS	
	Number	Length (in.)
$(NH_4)_2SO_4$	6.1 b[x/]	26.1 b
Urea	12.4 a	60.8 a
KNO_3 and $Ca(NO_3)_2$	12.3 a	59.6 a

[x/] $P \_ 0.05$

TABLE 8

EFFECT OF SOIL MIXTURE pH ON THE GROWTH OF LEUCOTHOE CATESBAEI

Initial pH	Final pH	GROWTH INDEX OF SHOOTS Number	Length (in.)
4	4.9	10.3 ns	46.9 ns
6	6.5	10.3	50.8

TABLE 9

EFFECT OF A NITRIFICATION INHIBITOR (2-chloro-6-trichloromethyl-pyridine) ON THE GROWTH OF LEUCOTHOE CATESBAEI

Treatment ppm	GROWTH INDEX OF SHOOTS	
	Number	Length (in.)
0	9.5 ns	50.8 ns
40	11.0	46.9

The leucothoe plants from the August-September study were over-wintered in an open cold frame to determine if the various treatments might affect their winter hardiness. All plants grown on NH_4-N died and only one grown on urea survived the winter. Eleven of sixteen plants grown on NO_3-N survived the winter and started to break bud after being brought into the greenhouse in March, 1972.

In this study there were several significant interactions between and among N source, pH and nitrification inhibitor and their effects on the number and length of shoots (Appendix, Tables 1, 2, 3 and 4).

Determination of NO_3-N in the Young and Old Leaves of Leucothoe from the August-September Experiment

Since NO_3-N had served as a suitable N source for the growth of leucothoe, leaf analysis was conducted to determine whether NO_3-N accumulation was affected by the source of N, the pH and the nitrification inhibitor. Young foliage (new growth resulting from the treatments) and old (foliage that was present before treatments were initiated) accumulated NO_3-N in similar amounts under all treatments (Table 10). However, those plants cultured on urea and NH_4-N accumulated twofold more NO_3-N than plants grown on NO_3-N. There was significantly greater NO_3-N accumulated in plants grown at pH 4 to 5 versus pH 6 to 7. The presence or absence of the nitrification inhibitor had no significant effects on the levels of NO_3-N in the tissue.

TABLE 10

EFFECT OF N SOURCE, pH AND NITRIFICATION INHIBITOR UPON THE NO_3-N CONTENT OF YOUNG AND OLD LEAF TISSUE OF LEUCOTHOE CATESBAEI

Treatment	NO_3-N, Percent Dry Weight	
	Young	Old
$(NH_4)_2SO_4$	.0275 a [x]	.0298 a
Urea	.0268 a	.0335 a
KNO_3 and $Ca(NO_3)_2$	.0148 b	.0198 b
pH 4	.0333 [y]	.0333 [y]
pH 6	.0128	.0210
Nitrification Inhibitor	.0232 ns [z]	.0318 ns
No Nitrification Inhibitor	.0233	.0228

[x] $P \leq 0.05$

[y] $P \leq 0.01$

[z] See Appendix - Tables 5 and 6

The NO_3-N and NH_4-N Content of Soils Used in the Culture of Leucothoe

The relative concentration of NO_3-N expressed as mg/pot was significantly affected by the source of N. Soils treated with NO_3-N carriers contained the greatest amounts of NO_3-N (Table 11), 28.1 mg, while urea and NH_4-N treated soils contained 12.4 and 3.6 mg N/pot, respectively. The pH and nitrification inhibitor did not significantly affect the levels of NO_3-N in the soil, but there were considerably greater amounts of NO_3-N in those soils not treated with the inhibitor (Table 11).

The NH_4-N concentration in the soil was also significantly affected by the source of N. Urea treated soils contained the greatest NH_4-N levels, followed by NH_4-N and NO_3-N treated soils, respectively (Table 11). Soils buffered at pH 6 to 7 contained fourfold greater NH_4-N levels than those buffered at pH 4 to 5 (Table 11). The nitrification inhibitor had no significant effect upon NH_4-N levels in the soils.

Nitrate Reductase Study

The fact that both leucothoe and Catawba rhododendron grew and developed on NO_3-N indicated that NO_3-N was assimilated by these plants. For NO_3-N to be of benefit to the plant it must be reduced to ammonia and then incorporated into glutamic acid via the glutamate dehydrogenase system. The reduction of NO_3^- to NO_2^- is the first step in this reductive process; the latter reduction of NO_2^- to NH_3 is catalyzed by nitrite reductase. Various investigators (38, 56, 64, 76, 79) have indicated that the two reductases are

TABLE 11

EFFECT OF N SOURCE, pH AND NITRIFICATION INHIBITOR UPON THE NO_3-N AND NH_4-N CONTENT OF SOILS USED IN CULTURE OF *LEUCOTHOE CATESBAEI*

	mg N/pot	
Treatment	NO_3-N	NH_4-N
$(NH_4)_2SO_4$	3.6 a [x]	6.0 a
Urea	12.4 b	16.1 b
KNO_3 and $Co(NO_3)_2$	28.1 c	2.7 c
pH 4	15.1 ns	13.2 [y]
pH 6	13.9	3.3
Nitrification Inhibitor	11.2 ns	9.0 ns
No Nitrification Inhibitor	18.3	7.5

[x] $P = 0.05$

[y] $P = 0.01$

in close association within the cell and that the complete reduction of NO_3^- to NH_3 usually occurs. Nitrite reductase activity (17) was shown to be significantly greater than nitrate reductase, and, consequently, NO_2^- never accumulates in plant tissue. If an active nitrate reductase system could be demonstrated in both leaves and roots of leucothoe, rhododendron, and other plants in the Ericaceae, then this would provide unequivocal evidence that these plants were indeed capable of utilizing NO_3-N for their growth and development.

Originally, the demonstration of nitrate reductase activity in ericaceous plants was confounded by the extraction method employed. Various investigators (34, 42, 78) indicated that the extraction procedure could have a profound influence on the recovery of active enzyme systems. The tissue homogenate method was used in the initial stages of this study as it is the standard extraction and assay technique used for demonstrating the presence of an active nitrate reductase fraction (41). However, no activity was detected in the leaves and roots of ericaceous plants and apple (Table 12). Activity in corn leaf extracts was excellent, but activity was not detected in root extracts.

Various modifications of this method failed to produce an active nitrate reductase fraction from the Ericaceae as well as apple and the roots of corn. The modifications are described under inhibitor studies.

A study was conducted to determine if the inactivation observed for nitrate reductase in ericaceous leaf extracts was also occurring with other enzyme systems. Leaf extracts of corn, leucothoe, Pieris and rhododendron were

TABLE 12

NITRATE REDUCTASE ACTIVITY IN LEAVES AND ROOTS OF SEVERAL SPECIES AS DETERMINED BY THE TISSUE HOMOGENATE METHOD

Species	mμ moles NO_2 produced/g fr wt/hr Leaves	Roots
Vaccinium corymbosum	0 ± [x]	0
Arctostaphylos Uva-ursi	0	0
Leucothoe catesbaei	0	0
Rhododendron catawbiense cv. Roseum Elegans	0	0
Kalmia latifolia	0	0
Oxydendrum arboreum	0	0
Pieris japonica	0	0
Malus sylvestris	0	0
Zea mays	2240 ± 70	0

[x] All values are the average of at least 5 determinations.

prepared as described in Materials and Methods and assayed for both malic and glutamic dehydrogenase activity. Malic dehydrogenase was active in corn leaf extracts, but was absent from the ericaceous leaf extracts (Figure 4). Glutamic dehydrogenase activity was also detected in corn leaf extracts but was absent in the ericaceous leaf extracts (Figure 5). This indicated the possibility of a non-specific inhibitor, that is, a compound which nonselectively reacts with and inactivates the protein during the homogenization process.

Tissue Infusion Method

The tissue infusion extraction and assay technique resulted in the demonstration of nitrate reductase activity in the leaves and roots of ericaceous plants as well as corn and apple (Table 13). Activity in roots of Ericaceae was between three- and seventeenfold greater than that observed in leaves (Table 13). The activity detected in apple leaf and root extracts was approximately equal while activity in corn leaf extracts was five- to sixfold greater than that of root extracts.

Nitrate reductase activity was determined in the young leaves of leucothoe which were grown on three N sources, at two pH levels and with and without a nitrification inhibitor as described under Greenhouse Experiments - August and September. Activity was significantly greater in urea-cultured plants versus NH_4-N or NO_3-N-cultured plants (Table 14). The pH of the growing medium and the presence or absence of the nitrification inhibitor had no significant effect on activity. Activity was also determined in the roots of water culture grown leucothoe and blueberry at various periods. These plants had been growing in

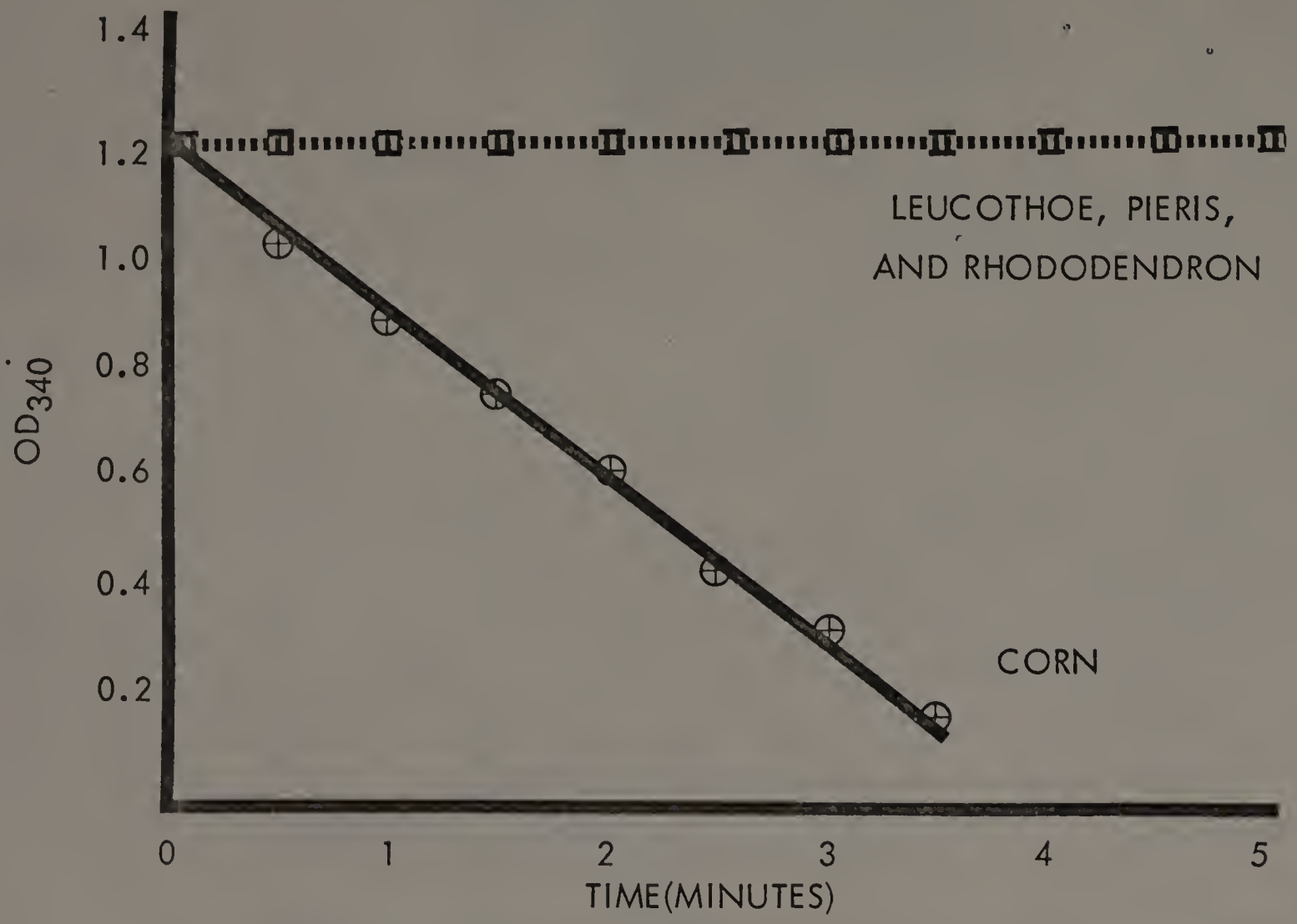

FIGURE 4

MALIC DEHYDROGENASE ACTIVITY IN CORN, LEUCOTHOE, PIERIS,
AND RHODODENDRON

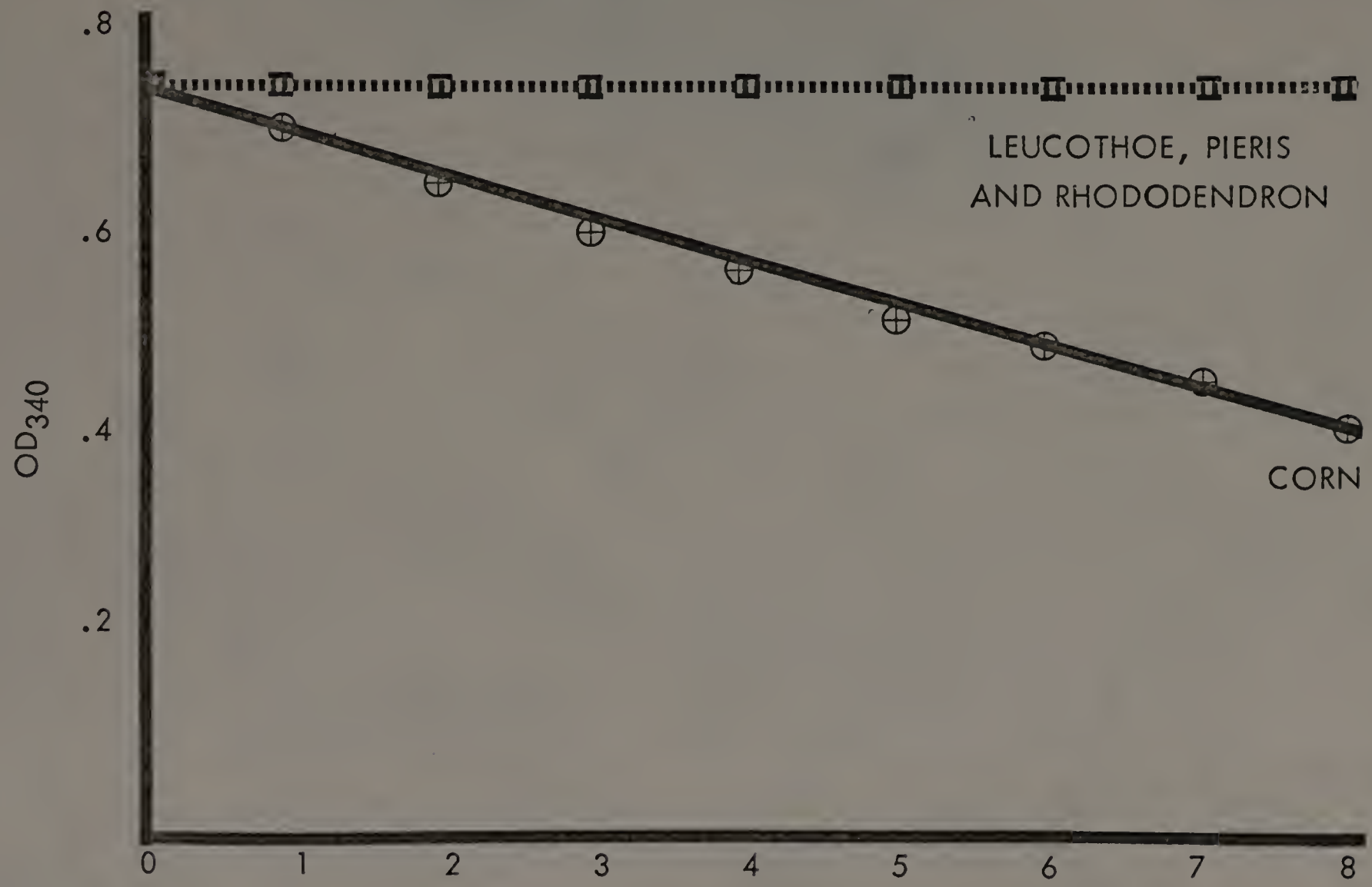

FIGURE 5

GLUTAMIC DEHYDROGENASE ACTIVITY IN CORN, LEUCOTHOE, PIERIS,

AND RHODODENDRON

TABLE 13

NITRATE REDUCTASE ACTIVITY IN LEAVES AND ROOTS OF SEVERAL SPECIES AS DETERMINED BY THE TISSUE INFUSION METHOD

Species	mμ moles NO_2 produced/g fr wt/hr Leaves	Roots
Vaccinium corymbosum	15 ± 5 [y]	260 ± 47
Arctostaphylos Uva-ursi	20 ± 5	75 ± 25
Leucothoe catesbaei	63 ± 13	395 ± 46
Rhododendron catawbiense cv. Roseum Elegans	20 ± 6	50 ± 5
Kalmia latifolia	120 ± 18	- [x]
Oxydendrum arboreum	13 ± 2	-
Rhododendron carolinianum	45 ± 8	-
Pieris japonica	16 ± 4	-
Malus sylvestris	122 ± 27	97 ± 8
Zea mays	750 ± 13	140 ± 36

[x] No nitrate reductase determinations were conducted with root tissue.

[y] All values are the average of at least 5 determinations.

TABLE 14

NITRATE REDUCTASE ACTIVITY IN THE LEAVES OF LEUCOTHOE CATESBAEI

Treatment	mμ moles NO_2^- produced/g fr wt/hr Activity ±[x]
$(NH_4)_2SO_4$	35 b [y]
Urea	48 a
KNO_3 and $Ca(NO_3)_2$	40 b
pH 4	44 ns
pH 6	39
Nitrification Inhibitor	45 ns
No Nitrification Inhibitor	37

[x] All values are the average of 4 determinations.

[y] $P \_ 0.05$

full strength Hoagland's (47) solution since 16 October 1971. Nitrate reductase activities of blueberry roots were 260 ± 47, 1410 ± 480 and 1750 ± 340 on 11 January 1972, 22 February 1972, and 25 April 1972, respectively; while activities in leucothoe root tissue were 395 ± 46 and 520 ± 60 on 11 January 1972 and 22 February 1972, respectively. Activity in leucothoe and blueberry leaf tissue was similar to that listed in Table 13.

Nitrate Reductase in Blueberry Leaves

The nitrate reductase activity detected in leaves of the 'Jersey' highbush blueberry is shown in Table 15. After 30 days of treatment, there were no significant differences in activity as a result of the various treatments; however, after 50 days there were significant differences. Plants cultured on NO_3-N at both pH 4 and 6 demonstrated significantly greater reductase activity than those cultured on NO_3-N plus NH_4-N or NH_4-N alone. Activity was also demonstrated in roots of plants cultured on NO_3-N at pH 4 and NH_4-N at pH 4. Activity was 48 ± 18 and 63 ± 20 mμ moles NO_2 produced/g fr wt/hr, respectively.

Induction Studies

The nitrate reductase active of leaf tissue incubated on nitrate was usually greater than that normally detected in leaf tissue grown under greenhouse conditions (Table 16 vs. Table 13 and 14). The activity in incubated Pieris and leucothoe was 130 and 182 mμ moles, respectively, while activity in

TABLE 15

NITRATE REDUCTASE IN LEAVES OF BLUEBERRY

N-Source	pH	Assay and Days of Treatment 30	50
		mμmoles NO_2 produced/g fr wt/hr	
NO_3	4	15.0 a ±/	15.0 c
$NO_3 + NH_4$	4	15.0 a	12.0 b
NH_4	4	15.0 a	11.0 b
NO_3	6	12.5 a	14.0 c
$NO_3 + NH_4$	6	12.7 a	8.3 a
NH_4	6	13.0 a	11.5 b

±/ All values are the average of 5 determinations.

Means followed by different letters are significantly different ($P \leq 0.05$ within columns).

TABLE 16

INDUCTION OF NITRATE REDUCTASE ACTIVITY IN LEAVES OF VARIOUS ERICACEOUS PLANTS INCUBATED IN A NITRATE MEDIUM WITH AND WITHOUT PROPANOL

	mμ moles NO_2 produced/g fr wt/hr	
Species	No Propanol	5% Propanol
Rhododendron carolinianum	45 ± 8 [x/]	45 ± 11
Rhododendron catawbiense cv. Roseum Elegans	26 ± 6	20 ± 5
Pieris japonica	73 ± 14	130 ± 33
Leucothoe catesbaei	130 ± 21	152 ± 20

[x/] All values are the average of 3 determinations.

incubated Pieris and leucothoe was 16 and 63 mμ moles, respectively. Activity in Pieris and leucothoe was much greater than that in either rhododendron species. The use of propanol was beneficial in stimulating activity in Pieris, but the other species demonstrated similar activity with and without propanol in the incubation medium. The relative amounts of NO_3-N were determined as this would indicate whether propanol was increasing NO_3-N movement and accumulation. The only large difference in NO_3-N content due to propanol treatment was in Carolina rhododendron where propanol treated tissue contained approximately twofold more NO_3-N than untreated tissue (Table 17). The other species exhibited no differences in NO_3-N content due to propanol. However, the relative amount of NO_3-N in the leaf pieces of leucothoe incubated in nitrate was six- to sevenfold greater than that of unincubated leaves (Table 10). Nitrate reductase activity could not be correlated with the NO_3-N in the leaf tissue, for although Pieris contained the same amount of NO_3-N with and without propanol treatment, activity was twofold greater in the propanol treated leaf pieces (Table 16). On the other hand, Carolina rhododendron contained twice the NO_3-N when incubated in propanol versus no propanol (Table 17); yet the nitrate reductase activity was the same (Table 16).

TABLE 17

NO_3-N CONTENT OF ERICACEOUS LEAF PIECES INCUBATED IN A NITRATE MEDIUM

Species	NO_3-N PERCENT DRY WEIGHT	
	No Propanol	5% Propanol
Rhododendron carolinianum	0.24	0.39
Rhododendron catawbiense cv. Roseum Elegans	0.40	0.40
Pieris japonica	0.21	0.20
Leucothoe catesbaei	0.16	0.16

Inhibitor Studies

Modifications of the Tissue Homogenate Method

Insoluble polyvinylpyrrolidone (PVP) added to leaf and root tissue during homogenization did not result in the extraction of an active nitrate reductase fraction from the leaves and roots of Kalmia, Leucothoe, Pieris, Rhododendron, Malus or roots of Zea (Table 18). Activity was detected only in corn leaf extracts, and the presence of PVP did not increase activity over those leaf extracts prepared without PVP (Table 18). To determine the effectiveness of PVP as a phenolic complexing agent the absorbancies of extracts prepared with and without PVP were determined. The data in Figure 6 show that blueberry and leucothoe extracts contained relatively more phenolic compounds than corn extracts in the absence of PVP. The leaf extracts of blueberry and leucothoe exhibited no nitrate reductase activity while corn extracts were extremely active (Table 18). Blueberry and leucothoe extracts prepared with PVP showed reduced absorbancies (Figure 6) indicating a lower phenolic content (4) yet exhibited no nitrate reductase activity (Table 18). There were no differences in the absorbancies of corn extracts prepared with and without PVP (Figure 6). Nitrate reductase activity in corn extracts was approximately the same in extracts prepared with and without PVP (Table 18).

Extracts of leucothoe and rhododendron prepared with and without PVP were dialyzed for 24 hours against large volumes of distilled water. The dialysate was then assayed for nitrate reductase activity. However, activity was not detected in either leucothoe or rhododendron extracts prepared with and without PVP (Table 19).

TABLE 18

NITRATE REDUCTASE IN LEAVES AND ROOTS OF SELECTED SPECIES AS DETERMINED BY MODIFICATIONS OF THE TISSUE HOMOGENATE METHOD

Species	Plant Part	Tissue Homogenate - PVP	Tissue Homogenate + PVP
		mμ moles NO_2^- produced/g fr wt/hr	
Kalmia latifolia L.	Leaves	0	0
Leucothoe catesbaei Gray	Leaves	0	0
Leucothoe catesbaei Gray	Roots	0	0
Malus sylvestris Mill. McIntosh	Leaves	0	0
Pieris japonica D. Don.	Leaves	0	0
Rhododendron catawbiense Michx. Roseum Elegans	Leaves	0	0
Vaccinium corymbosum L.	Leaves	0	0
Zea mays L.	Leaves	2240 ± 70	2240 ± 70
Zea mays L.	Roots	0	0

Figure 6. Absorbance of extracts of homogenized corn, blueberry, and leucothoe leaves. Leaves were ground in 0.2M tris buffer, pH 7.5, and diluted 100:1 with 0.1M phosphate buffer, pH 7.0. The absorbancies from 260 nm to 340 nm indicate the relative amounts of various phenolic compounds in the extracts (4, 77). LEFT: Without polyvinylpyrrolidone (PVP) added during the homogenization and extraction. RIGHT: 3 g of hydrated (Klepper and Hageman 54) PVP per g fr. wt. of tissue added during homogenization and extraction.

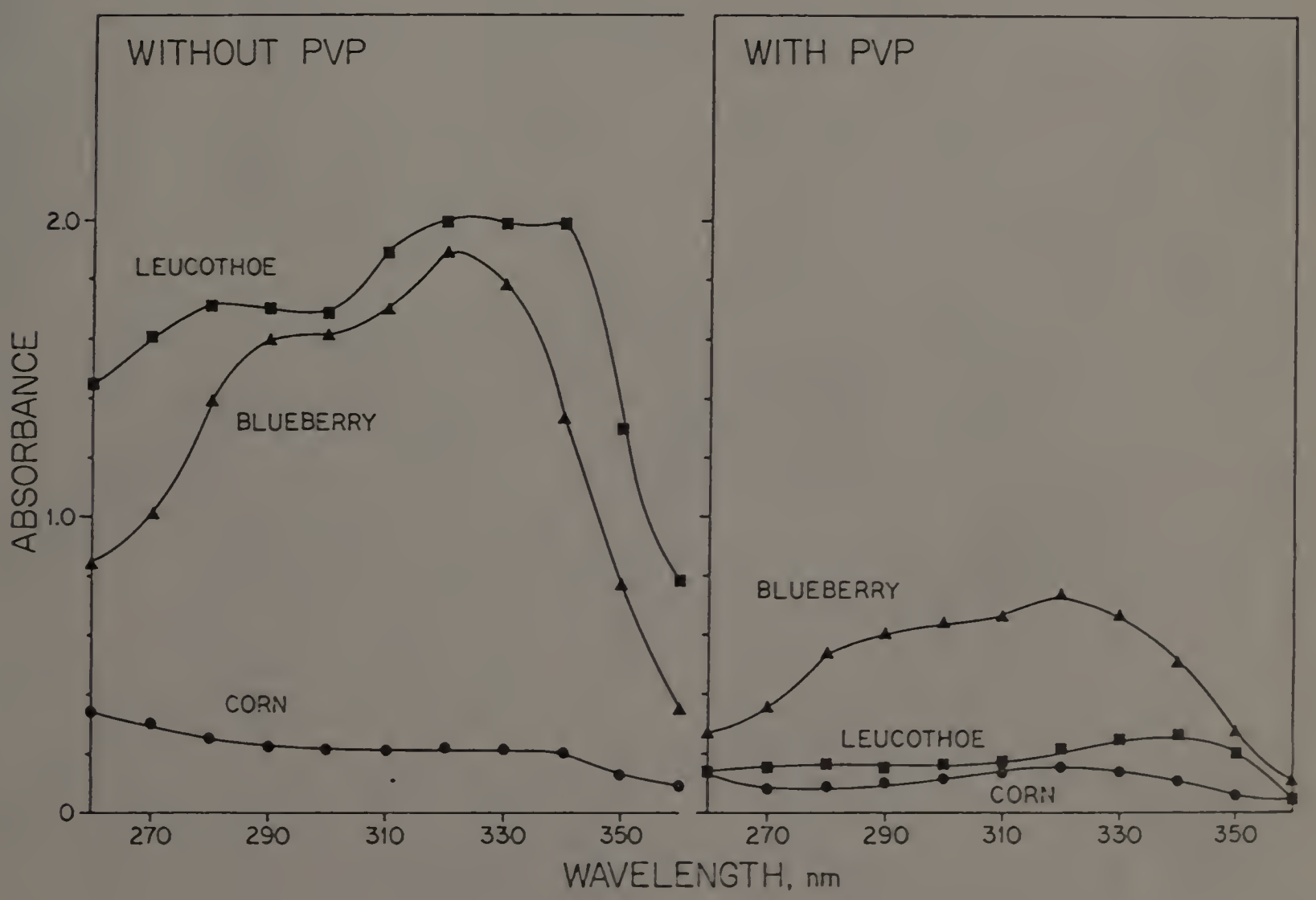

TABLE 19

NITRATE REDUCTASE IN LEAF EXTRACTS OF LEUCOTHOE AND CATAWBA RHODODENDRON PREPARED WITH AND WITHOUT PVP AND THEN SUBJECTED TO DIALYSIS

Species	Activity	
	- PVP	+ PVP
	mμmoles NO_2^- produced/g fr wt/hr	
Dialyzed Leucothoe Extracts	0	0
Dialyzed Rhododendron Extracts	0	0

Characterization of Inhibitor

Fresh extracts of leucothoe and rhododendron were added back to active corn extracts in equal concentrations (0.5 ml ericaceous extract to 0.5 ml corn extract) to determine if the inhibitors that were affecting the extraction of an active nitrate reductase fraction from ericaceous plants would affect corn leaf extract activity. Activity in corn extracts was severely inhibited by the ericaceous leaf extracts (Figure 7). The inhibitory substances in rhododendron were extremely potent, for at a concentration of 0.01 ml of fresh extract added to 0.5 ml of active corn extract, nitrate reductase activity was reduced 77%. Leucothoe also possessed a potent inhibitory substance as 0.2 ml to 0.5 ml of active corn extract resulted in a 96% reduction in activity (Figure 7).

Leucothoe and rhododendron extracts prepared with and without PVP were added back to active corn extracts at equal concentrations as described above. As previously demonstrated (Table 18 and Figure 6), PVP did not result in the extraction of an active nitrate reductase, but did reduce the phenolic content. Extracts prepared without PVP were completely inhibitory while those prepared with PVP resulted in 50% inhibition (Table 20). This indicated that phenolic compounds were partially responsible for the inhibition.

Boiled leaf extracts of the two ericaceous species prepared with and without PVP and added back to active corn leaf extracts were inhibitory in the same manner as fresh extracts (Table 21). Extracts prepared without PVP were completely inhibitory while those prepared with PVP reduced activity 50% of control. This was strong evidence that the inhibitors were not proteinaceous and were not heat labile.

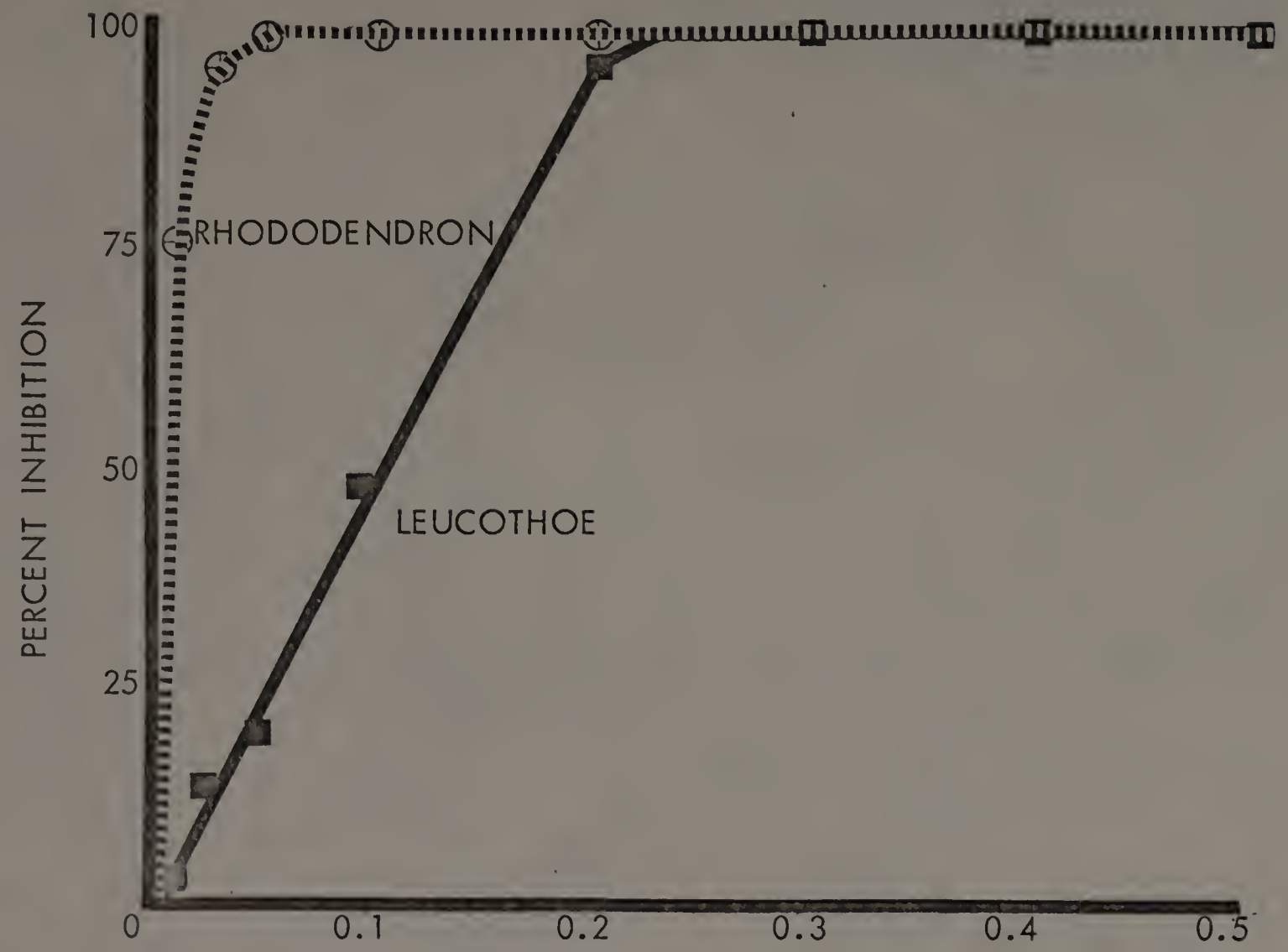

FIGURE 7

EFFECT OF RHODODENDRON AND LEUCOTHOE EXTRACTS UPON THE NITRATE REDUCTASE ACTIVITY IN ACTIVE CORN EXTRACTS

TABLE 20

INHIBITION TO NITRATE REDUCTASE FROM CORN LEAVES BY FRESH EXTRACTS OF LEUCOTHOE AND RHODODENDRON PREPARED WITH AND WITHOUT PVP

Species	Treatment	% Inhibition
Leucothoe	- PVP	100 x/
	+ PVP	50
Rhododendron	- PVP	100
	+ PVP	50

x/ Average value of over 25 determinations.

TABLE 21

INHIBITION TO NITRATE REDUCTASE FROM CORN LEAVES BY BOILED EXTRACTS OF LEUCOTHOE AND RHODODENDRON PREPARED WITH AND WITHOUT PVP

Species	Treatment	% Inhibition
Leucothoe	- PVP and boiled extract	100 x/
Rhododendron		100
Leucothoe	+ PVP and boiled extract	50
Rhododendron		50

x/ Average values of 10 observations.

Extracts of leucothoe and rhododendron which had been stored on the laboratory bench for seven days were added to active corn extracts. The extracts were completely inhibitory to corn nitrate reductase activity indicating the inhibitors were very stable with time.

Fresh and boiled dialyzed extracts of leucothoe and rhododendron prepared with and without PVP were added to active corn extracts and the percent inhibition determined. Dialyzed fresh and boiled extracts of both ericaceous species prepared without PVP were completely inhibitory (Table 22). The leucothoe extracts prepared with PVP inhibited nitrate reductase activity 30%. These results indicated that some of the inhibition might be caused by a small micromolecular compound but that the greatest part of the inhibition was due to a large molecular species.

The resuspended leucothoe and rhododendron concentrates (as described under Fractionation Study) were completely inhibitory to active corn extracts (Table 23), while the resuspended ericaceous acetone pellet which supposedly contained the protein fraction had no effect on activity (Table 23). The resuspended ericaceous concentrates were then subjected to saturating amounts of PVP. The PVP treated extracts reduced activity by approximately 20% indicating phenolic compounds were largely responsible for the inhibition. The resuspended acetone pellet was analyzed for protein content by the biuret (28) method. No protein was detected indicating that the protein fraction could have been lost in the initial homogenization step.

TABLE 22

INHIBITION OF NITRATE REDUCTASE FROM CORN LEAVES BY FRESH AND BOILED DIALYZED EXTRACTS OF LEUCOTHOE AND RHODODENDRON PREPARED WITH AND WITHOUT PVP

Species	Treatment	% Inhibition
Leucothoe	- PVP and dialyzed boiled extract	100
Rhododendron		100
Leucothoe	- PVP and dialyzed fresh extract	100
Rhododendron		100
Leucothoe	- PVP and dialyzed boiled extract	30
Rhododendron		50
Leucothoe	- PVP and dialyzed fresh extract	30
Rhododendron		50

TABLE 23

INHIBITION OF NITRATE REDUCTASE FROM CORN LEAVES BY RESUSPENDED CONCENTRATES AND ACETONE PELLETS OF LEUCOTHOE AND RHODODENDRON

Species	Treatment	% Inhibition
Leucothoe	Resuspended concentrate x/	100 y/
Rhododendron		100
Leucothoe	Resuspended acetone pellet	0
Rhododendron		0
Leucothoe	Resuspended concentrate plus	18
Rhododendron	saturating amounts of PVP	20

x/ Preparation of the concentrates and pellets described in Fractionation Study.

y/ All values are the average of 9 determinations.

These results suggest that the inhibitor could be characterized as soluble, nonproteinaceous, phenolic and of a nondialyzable size. Furthermore the evidence suggested that the specific phenolics were 'tannin-like' in nature due to their particular properties (89). Andersen (4) indicated that absorbancies of plant extracts provided a qualitative index to the level of phenolics. The absorbancies of the resuspended ericaceous concentrates treated with and without PVP, of resuspended protein pellets and corn extracts treated with and without PVP were determined. The leucothoe resuspended concentrate and rhododendron resuspended concentrate were high in phenolic compounds, (Figures 8 and 9), respectively. The protein extracts demonstrated negligible absorbancies (Figures 8 and 9). Absorbancies of PVP treated ericaceous concentrates were twenty-fivefold less than non-PVP treated ericaceous concentrates. Corn extracts treated with PVP exhibited no difference in absorbancy from non-PVP treated extracts. Qualitatively, this indicated that a relatively low level of phenolic compounds were present in corn.

A 'tannin-like' compound was seemingly responsible for the inhibition to active corn nitrate reductase as well as the inactivation of nitrate reductase from ericaceous plants during homogenization. Therefore, various simple phenols, hydrolyzable tannins, and tannic acid were added back to active corn extracts to determine which group of compounds was actually inhibitory. The only compound to have any effect was tannic acid which is a mixture of gallic acid and various galloyl esters of glucose (Table 24). The simple phenolics and hydrolyzable tannins had no effect on nitrate reductase activity in corn. Tannic acid at 10^{-4}

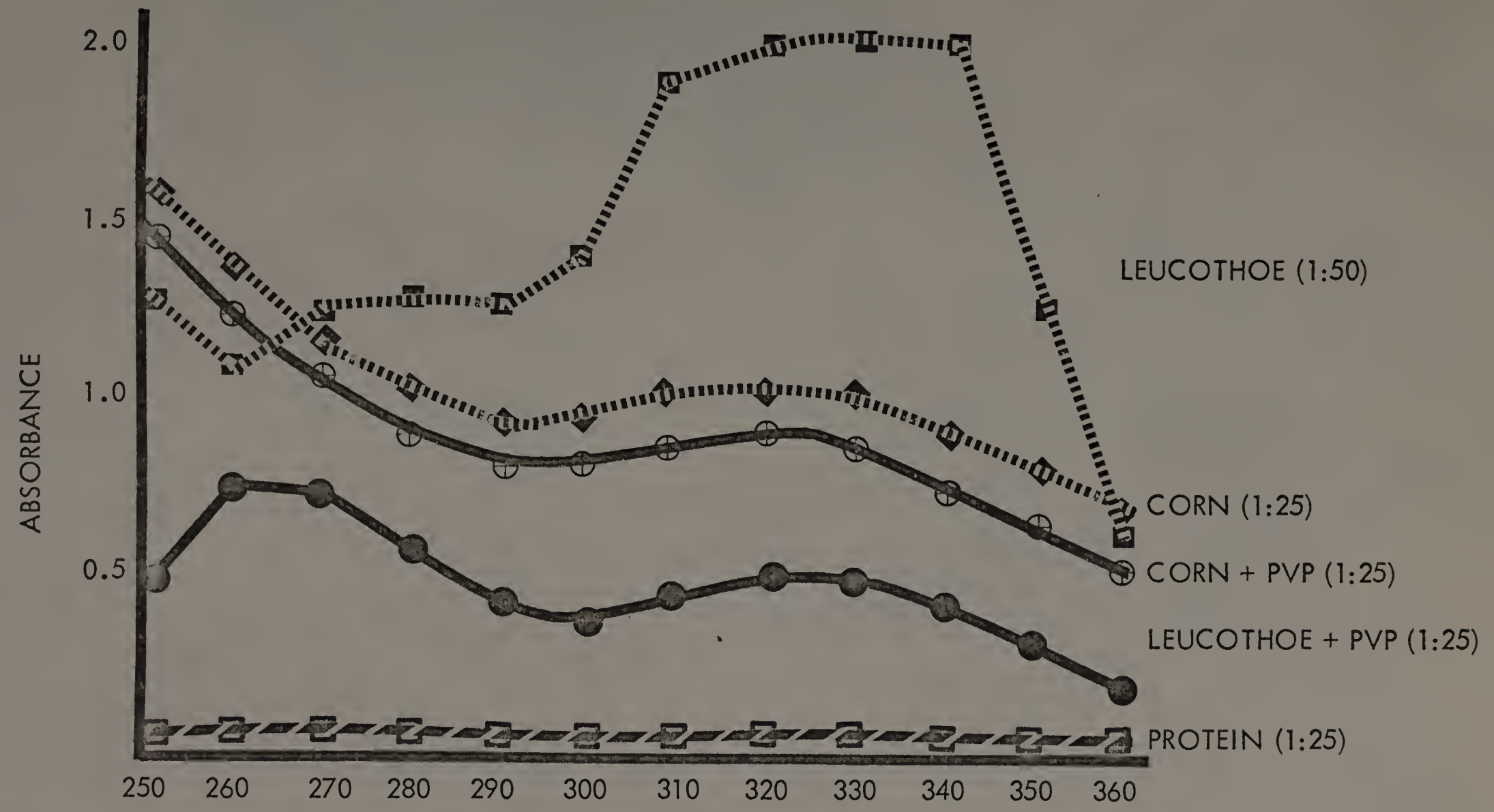

FIGURE 8

ABSORBANCIES OF LEUCOTHOE AND CORN EXTRACTS

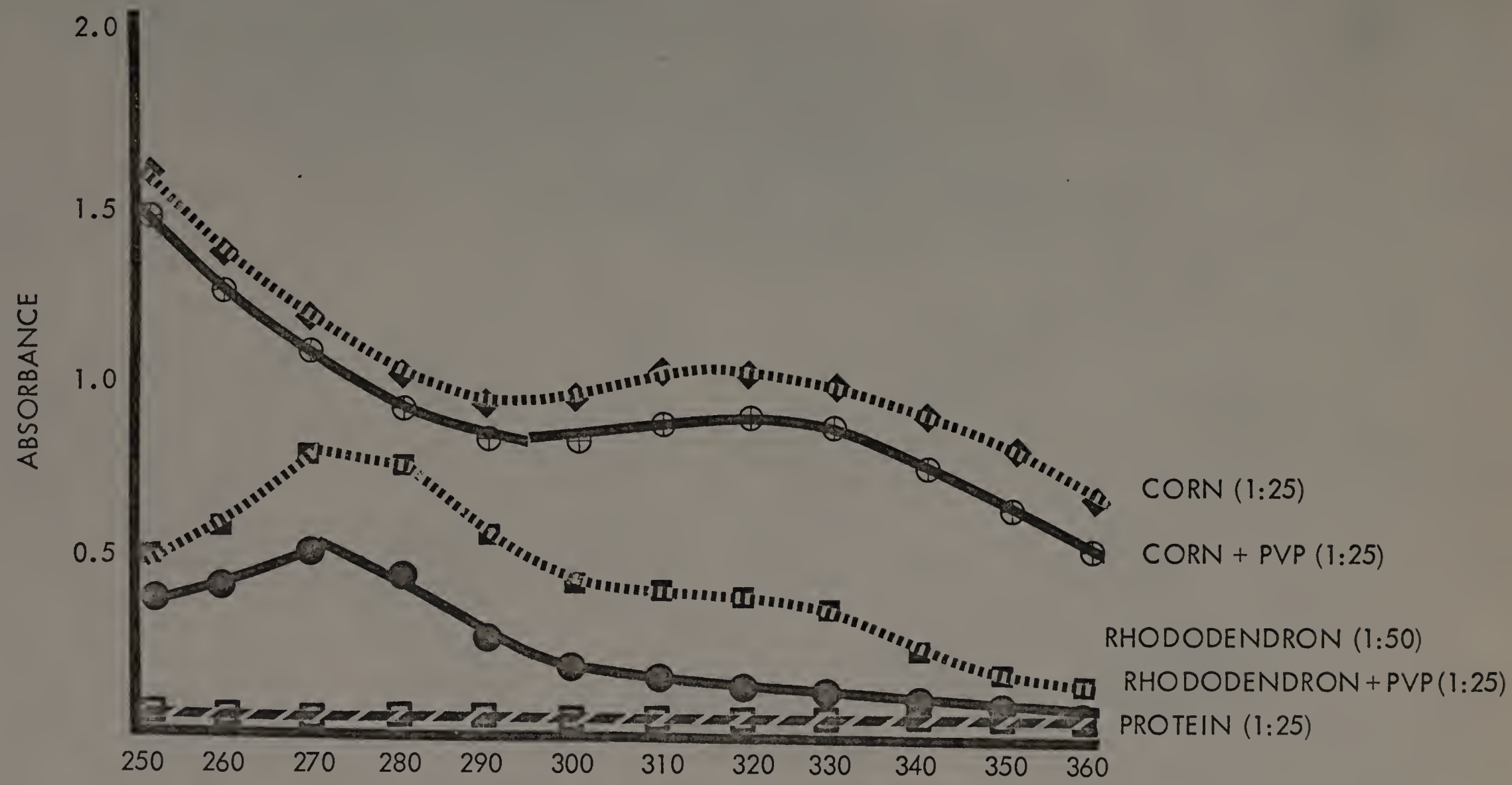

FIGURE 9

ABSORBANCIES OF RHODODENDRON AND CORN EXTRACTS

TABLE 24

THE EFFECT OF VARIOUS PHENOLIC COMPOUNDS ON THE NITRATE REDUCTASE ACTIVITY OF ACTIVE CORN EXTRACTS

Compound	Relative Inhibition or Promotion of Nitrate Reductase Activity in Corn
Quinic Acid x/	No Effect
Juglone	No Effect
Catechin	No Effect
Chlorogenic Acid	No Effect
Hydroquinone	No Effect
Tannic Acid	Complete Inhibition
Tannic Acid and Polyclar AT	No Effect
Pyrogallol	No Effect
Rutin	No Effect

x/ The relative effects are the result of 9 observations with each phenolic.

and 10^{-5} M was 100% and 15% inhibitory, respectively, to corn nitrate reductase activity. When PVP was added to tannic acid (10^{-3} M) inhibition was completely removed (Table 24).

Concentrates of leucothoe and rhododendron prepared with and without PVP were spotted with tannic acid and chlorogenic compounds for reference values. Upon examination under ultraviolet light extracts prepared without PVP showed a distinct band which coincided with both the known tannic acid and chlorogenic fraction. Extracts prepared with PVP showed reduced fluoresence and there was no band corresponding to the known tannic acid or chlorogenic acid. The use of $FeCl_3$ and $FeNH_4(SO_4)_2$ both gave positive tests for galloyl tannins when applied to the non-PVP treated extracts. However, those extracts treated with PVP and chromatographed showed no colorimetric response.

Bacterial and Mycorrhizal Study

Bacterial Study

Bacteria are capable of reducing NO_3-N to NO_2-N (54). The fact that activity in leaf tissue was low (Table 13) indicated that possibly bacteria might in some way be influencing the observed activity. However, bacterial contamination was not found in leucothoe leaf pieces washed in the 2% and 4% hypochlorite solutions, while those washed in distilled water were contaminated (Figure 10). Leaf pieces treated in a similar manner were then utilized for nitrate reductase determinations. There were no significant differences in activity among the various treatments (Table 25).

FIGURE 10. EFFECT OF DISTILLED WATER, 2% AND 4% HYPOCHLORITE WASHES ON THE BACTERIAL CONTAMINATION OF LEUCOTHOE LEAF PIECES.

TABLE 25

EFFECT OF DISTILLED WATER AND 2% AND 4% HYPOCHLORITE WASHES ON THE NITRATE REDUCTASE ACTIVITY IN LEUCOTHOE LEAF SECTIONS

Treatment	Activity
	mμ moles NO_2 produced/g fr wt/hr
Leaves removed directly from plant	35 a
Leaves washed in distilled water	28 a
Leaves washed in 2% hypochlorite	38 a

Chloramphenicol (50 µg/ml) was added to the incubating leaf sections and the nitrate reductase activity of these sections was compared with that of untreated leaf sections. There were no large differences between the two treatments, again indicating that bacteria had no effect on the observed nitrate reductase activity.

Mycorrhizal Study

Fungi are capable of reducing NO_3-N to NO_2-N (33, 54) and; therefore, a study was conducted to determine if mycorrhizae were present in the roots of ericaceous plants. There was no evidence of fungal infection in the roots of greenhouse grown Ericaceae (Figures 11 A, 11 B, 11 C and 11 D). Root samples collected from a wild highbush growing in relatively infertile soil possessed mycorrhizal infection (Figures 12 A, 12 B, 12 C and 12 D). Evidence that the mycorrhizae were endotrophic is shown in Figure 12 D where the hyphal strands are actually inside the cells.

EXPLANATION OF FIGURES

Figure 11 A — Uninfected roots of soil grown Catawba rhododendron cultured under high nutritional status. Roots are very fine in structure and there is no indication of infection similar to that found in the blueberry roots (X 25).

Figure 11 B — Uninfected roots of soil grown leucothoe. Roots are very small and thread-like in appearance (X 25).

Figure 11 C — Uninfected roots of water culture grown leucothoe (X 25).

Figure 11 D — Uninfected roots of water culture grown highbush blueberry (X 25).

Figure 12 A — Infected roots of highbush blueberry which were collected from the wild. Masses of fungal hyphae appear as swollen areas along the root axis (X 25).

Figure 12 B — Infected root of highbush blueberry showing dense hyphal mass. Darkened area in center of infected root is the vascular tissue of the blueberry root. Root hair-like projections emanating from the infected root are also distinctly noticeable (X 25).

Figure 12 C — Close-up of the root hair-like projections. These structures are not root hairs, but part of the fungal hyphae. Morphologically (52) they resemble root hairs (X 100).

Figure 12 D — Close-up of hyphae within the blueberry root cells. The hyphae appear as coiled, thread-like substances (X 100).

FIGURE 11 A

FIGURE 11 B

FIGURE 11 C

FIGURE 11 D

FIGURE 12 A

FIGURE 12 B

FIGURE 12 C

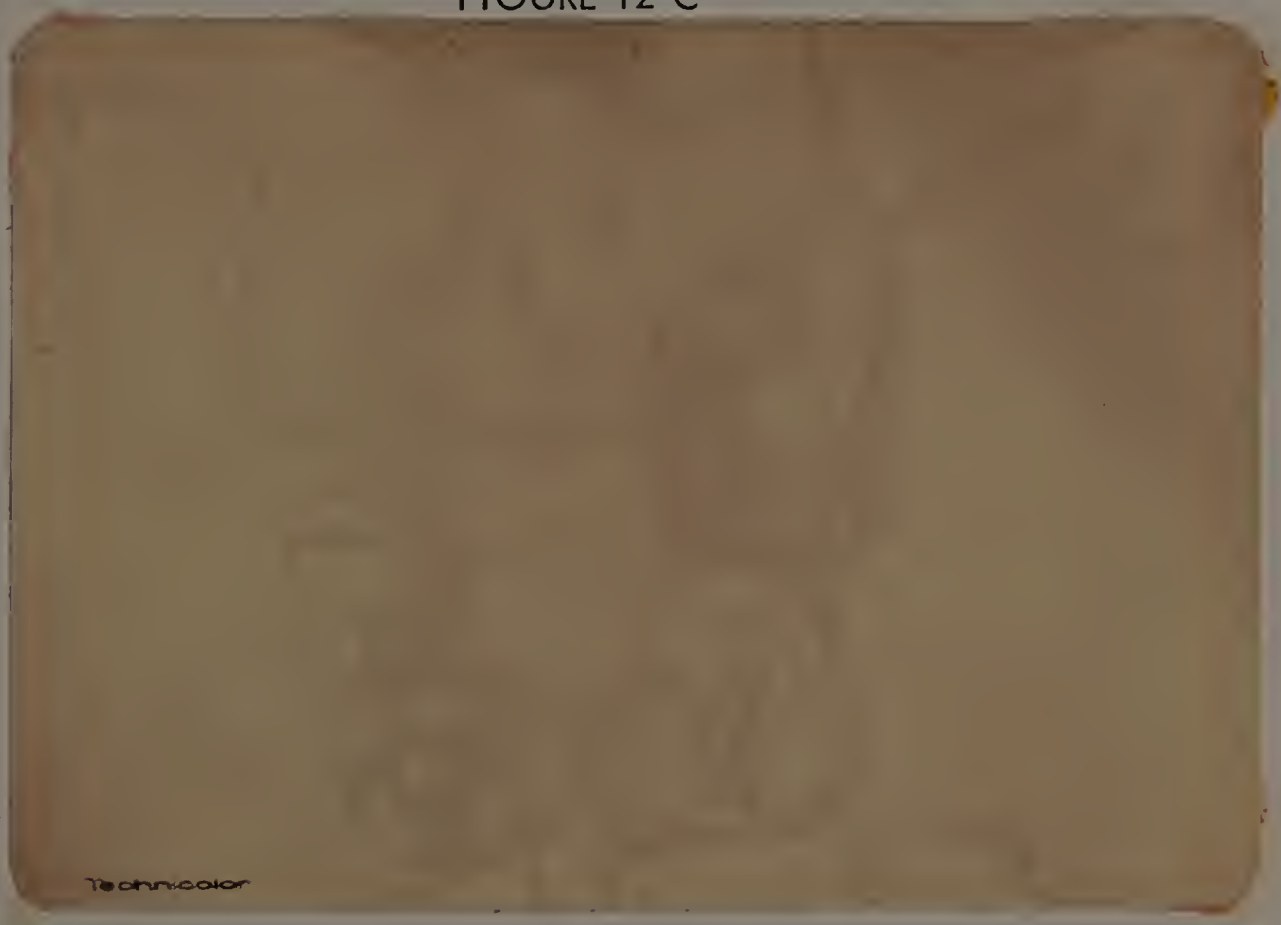

FIGURE 12 D

DISCUSSION

Greenhouse Experiments

February and March Leucothoe and Rhododendron Studies

The results indicated that NO_3-N was manifestly superior to either urea or NH_4-N in supporting the growth and development of these two ericaceous species. This was significant in view of the previous literature concerning the N nutrition of the Ericaceae (1, 2, 8, 19, 29, 40, 46, 50, 82, 91, 93, 94). Nitrate N did not result in chlorotic conditions in either leucothoe or rhododendron as was previously reported with other Ericaceae (19, 91). The fact that iron was present as the chelate in sufficient concentrations compensated for the high pH range, as Oertli (70) demonstrated excellent growth of blueberries and azaleas at pH levels of 7 or greater provided iron was present as the chelate. He found that $FeCl_3$ did not prevent chlorosis at the high pH. Leucothoe and rhododendron cultured at pH 6 to 7 showed as good or better vegetative growth than plants cultured at pH 4 to 5 which is difficult to explain in view of reported effects of pH on the growth of the Ericaceae (10, 23, 25, 45, 97, 99). Possibly the lower pH range affected the availability of some of the essential elements as it has been shown that Ca, Mg, P, K and S are less available at lower pH ranges (22). On the other hand, these elements are maximally available to a plant in the pH range 6 to 7, and this could partially explain the excellent growth at this pH range. The fact that the nitrification inhibitor was of no

benefit in supporting growth was further evidence that NH_4-N was not the most suitable form of N for leucothoe and rhododendron. Also the necrosis appeared only when urea or NH_4-N was applied to the plants indicating that ammoniacal N sources can be detrimental if applied continuously.

August and September Leucothoe Study

The results of this study agreed with the results of the February and March study. Urea produced vegetative growth comparable to NO_3-N (Table 7), but resulted in severe necrosis (Figure 1) and this would eliminate its usage as a fertilizer for these plants. Ammonium N was ineffective in stimulating vegetative growth of leucothoe and resulted in necrosis (Table 7 and Figure 2). Ammonium N can affect respiration (82), uncouple photosynthetic phosphorylation (60), result in a degeneration of chloroplastic membranes (74) and affect other plant processes (12, 83). Since energy production is curtailed, subsequent energy dependent reactions are disrupted and death of cells occurs. To unequivocally state that ammoniacal N sources should never be employed in the fertilization of woody ornamental Ericaceae is unjust. Undoubtedly, reasonably good growth could have been obtained from the ammoniacal N source if it had been applied in low concentrations and at less frequent intervals than were employed in these studies. Townsend (91) demonstrated that NH_4-N concentrations as low as 14 ppm supplied three times weekly produced excellent growth of the lowbush blueberry grown in sand culture. However, plants supplied with NH_4-N at 140 ppm three times weekly developed necrosis similar to that which

was observed on leucothoe and rhododendron. This was additional evidence that NH_4-N can be detrimental in high concentrations to an ericaceous species.

Nitrate Nitrogen Content of Leucothoe and NO_3-N and NH_4-N Content of Soils

The NO_3-N content of leucothoe under all treatment conditions (Table 10) was extremely low when compared to other species (13). The fact that young and old leaf tissue contained approximately the same concentrations of NO_3-N was strange in view of the fact that metabolism should be lower in an old leaf, and, therefore, more NO_3-N should accumulate. The actual explanation as to why there was significantly more NO_3-N in urea and NH_4-N grown plants is difficult to explain. Barker and Maynard (13) have shown that the NO_3-N content of cucumber and pea shoots increased as the NO_3-N content of the solution culture increased. It is also difficult to explain why the NO_3-N concentrations in young and old leaf tissue from plants grown at pH 4 to 5 was significantly greater than in leaf tissue from plants cultured at pH 6 to 7 (Table 10) in view of the fact that there were no significant differences in the soil NO_3-N levels at the two pH ranges (Table 11). The nitrification inhibitor did not affect the NO_3-N levels in the leaf tissue (Table 10) thus providing further evidence that its usage was unwarranted.

Nitrate N was present in the largest quantity in the soil when the nitrogen carriers were KNO_3 and $Ca(NO_3)_2$. Unfortunately, the amounts in the soil (Table 11) did not correlate with the foliage NO_3-N (Table 10). Although there were no significant differences in NO_3-N due to the presence or absence

of the nitrification inhibitor, there were considerable amounts of NO_3-N in soils not treated with the nitrification inhibitor which was expected since the inhibitor prevents the oxidation of NH_4-N to NO_3-N.

Ammonium N in soils was greatest with urea nutrition. The NH_4-N extracted from the urea treated soils was not due to the hydrolysis of urea during the distillation process, for commercially prepared urea was subjected to the distillation process and in no case was NH_4-N detected in the receiving flasks. According to Alexander (3) the conversion of urea to ammonium in soil far exceeds the rate of nitrate appearance so that ammonium continues to accumulate as long as urea is present. The fact that soil NH_4-N was approximately fourfold greater at pH 4 to 5 than 6 to 7 (Table 11) suggested that ammonia volatilization might have occurred at the higher pH range and also that the ammonium oxidizing bacteria were active which is consistent with previous reports (3). NH_4-N levels were essentially similar whether a nitrification inhibitor was present or absent. This indicated that possibly the inhibitor was not particularly effective in eliminating the Nitrosomonas bacteria which catalyze the oxidation of NH_4-N to NO_2-N.

Nitrate Reductase Study

The nitrate reductase system was never extracted in an active form from ericaceous plants when the tissue homogenate was utilized (Table 12). The tissue homogenate method involves grinding leaf or root tissue in a buffered medium and utilizing the supernatant as the source of crude enzyme. Many plants contain substances which have been shown to be inhibitory to various enzyme

systems (104). The most notable protein inactivators are the phenolics, especially those classified as tannins (89). The extraction of viable enzymes (62) and active mitochondrial fractions (49) has been shown to be dependent on keeping the phenolics in a reduced state during extraction and in some fashion complexing them irreversibly before they inactivate the protein. Loomis (62) and Anderson (5) have reviewed the literature concerning enzyme extraction and protection against phenolic compounds. Ericaceous plants are abundantly supplied with phenolics (80) and more specifically tannins (67), and, therefore, the extraction of an active nitrate reductase by the tissue homogenate methodology was unsuccessful.

The fact that active fractions of malic and glutamic dehydrogenase were not extracted from either leucothoe or rhododendron was further circumstantial evidence that the inhibitor was 'tannin-like' in nature since these compounds are nonspecific inactivators of protein (89).

The tissue infusion method, with which nitrate reductase activity was detected in all species (Table 13), is an in vivo method and the activity demonstrated with this type of method may be analogous to the rates of reduction occurring in the living plant (42). With this system there is a minimal disruption of vacuoles and other cellular compartments where phenolics are naturally stored, and, consequently, the protein fraction remains viable and active. Since this system is dependent on the rate of diffusion, nitrite reduction, and environmental factors, the observed rates of nitrate reduction are not as high as those demonstrated with the tissue homogenate method (Table 12 vs. Table 13). Klepper (56) indicated that the in vivo system which he employed resulted in three- to twentyfold lower

nitrate reductase activity than that detected by the homogenate method in several species. He also indicated that in other species activity was demonstrable only when the in vivo extraction and assay technique was employed. Lower rates of activity with the infusion method occur because NO_3-N must diffuse into the cytoplasm, be reduced and released from the enzyme, and diffuse back out into the incubation medium; also, nitrite reduction is probably occurring at a slow rate and thus utilizes some of the NO_2-N. Klepper (56) has shown that if the system was continuously illuminated then NO_2-N was never found in the incubation medium indicating that the NO_2-N was reduced to NH_3. However, when the incubation medium was kept in the dark, NO_2-N levels were very high indicating that nitrite reductase was not functioning. This light-dark effect on nitrite accumulation in the in vivo system can be explained on the basis that light produced a reductant necessary for the continued operation of the nitrite reductase system; however, when light was excluded the reductant was no longer produced and nitrite reduction was slowed down or stopped. On the other hand, the tremendous activity in the homogenate method is the result of the intimate association of enzyme-substrate-cofactor in a buffered solution maximal for nitrate reductase activity. Also the absence of nitrite reducing system eliminates the possibility that NO_2-N could be lost by further reduction.

The demonstration of activity in leaf and root tissue of ericaceous species (Table 13) supported the hypothesis that these plants were capable of utilizing NO_3-N. Activity was greatest in the roots in all cases (Table 13) indicating

that the bulk of the reduction occurred in this organ and that possibly the reduced nitrogen was translocated to the shoot in an organic form. Nitrate reductase activity in the leaf tissue of leucothoe and blueberry was low and the NO_3-N content extremely low, suggesting that the quantities of NO_3-N passing the root nitrate reductase system were too low to induce the enzyme in the leaf (42) to the same degree as in the root. Hageman (42) agreed with this interpretation.

The induction studies were important from several aspects in that they answered some of the questions from the previous work. Especially the question of whether increased tissue NO_3-N would result in increased activity was answered by this study. The activity in the incubated leaf tissue (Table 16) was usually much greater than that from plants grown in soil culture (Table 13). Ferrari (36) suggested that propanol increased the movement of NO_3-N into the cytoplasm. However, in this study propanol had no large effect on either nitrate reductase activity (Table 16) or NO_3-N content (Table 17). The relative amounts of leaf tissue NO_3-N were much greater than that normally found in the Ericaceae (Table 17 vs. Table 10). This indicated that these plants were capable of accumulating NO_3-N and also supported the contention that the reason for the low activity in leucothoe leaves could have been due to the low amounts of NO_3-N. Unfortunately, as can be seen from Tables 16 and 17, the NO_3-N content of the tissue did not correlate with the nitrate reductase activity. Other investigators have noted this anomaly (13).

The nitrate reductase activity in greenhouse grown leucothoe was essentially the same under all treatments (Table 14). Evans (34) stated that a small amount of NO_3-N can induce nitrate reductase and the fact that there were adequate amounts of NO_3-N in leaves and soil (Tables 10 and 11) of all treatments could explain the reason for the similarities in nitrate reductase activity. He also indicated that in order to demonstrate absolute differences in activity among treatments a sterile system such as that employed in the induction studies must be employed.

The presence of nitrate reductase in blueberry leaves indicated that these plants were capable of developing on NO_3-N (Table 15). Although activity was low it was present under all treatments. Although no growth measurements were made it appeared that growth was equivalent under all N regimes and pH levels indicating that if NH_4-N is kept at a low level toxicity problems do not arise. The pH per se had no direct effect on growth if iron and other readily inactivated elements were provided in adequate amounts. Nitrate reductase activity in roots was higher than that observed in leaf tissue indicating that the bulk of the reduction occurred in the roots. The actual amount of NO_3-N in leaf tissue was only 0.007 per cent on a dry weight basis. This was considerably less than that in greenhouse grown leucothoe (Table 10) or the induced leaf tissue pieces (Table 17). The death of plants cultured on NH_4-N plus NO_3-N treatments was surprising since plants cultured on NH_4-N or NO_3-N were not affected.

Inhibitor Study

The presence of PVP did not result in the extraction of an active nitrate reductase from the Ericaceae and apple roots and leaves, or corn roots. This did not agree with Klepper's (57) work with apple nitrate reductase, Loomis and Battaile's (62) work with mint enzymes, and Andersen's (4) work with tobacco enzymes. Klepper (57) was able to demonstrate nitrate reductase activity in leaves and roots of apple when PVP was added in the homogenization process. If PVP was not used, an active nitrate reductase fraction was not extracted. PVP was shown to optimally bind phenolics around pH 5.5 (4), yet Klepper's (57) extraction buffer was pH 8.8 and he received beneficial effects from PVP. The buffer used in this study was maintained at pH 7.5, thus it seemed that an active protein fraction should have been extracted.

There was no doubt that PVP was complexing the phenolic compounds. The absorbancy data with blueberry and leucothoe extracts (Figure 6) positively indicated that phenolic compounds were complexed and removed from solution by PVP. However, Andersen (4) has shown that after three successive treatments with PVP, phenolics still remained in solution.

Dialyzed extracts of leucothoe and rhododendron did not contain a viable nitrate reductase indicating that inhibitory substances were nondialyzable. Grasmanis (39) was able to demonstrate an active nitrate reductase in apple root extracts provided the extracts were first dialyzed for long periods of time. The failure to demonstrate nitrate reductase activity in leucothoe and rhodendron after 48 hrs of dialysis indicated that the inactivation could have occurred in the initial homogenization step.

Leucothoe and rhododendron extracts completely inhibited nitrate reduction in corn extracts indicating that the inhibitor was relatively stable and soluble. Even more striking was the fact that 0.01 ml of rhododendron extract added to 0.5 ml of active corn extract inhibited nitrate reductase activity by 77%. This was evidence that the compounds were inhibitory in relatively low concentrations. The fact that leucothoe extracts were not as inhibitory to corn extracts (Figure 7) indicated the inhibitors were not of the same nature or were present in lower concentrations.

Ericaceous extracts prepared with PVP according to the directions of Klepper (57) inhibited the nitrate reductase activity in corn extracts by 50% indicating that phenolics were responsible for a percentage of the observed inhibition. This 50% value represents the average of many observations and the actual inhibition values ranged from 40% to 60%. Leucothoe extracts usually caused the least inhibition while rhododendron extracts resulted in the higher inhibition values. The reasons for the scattering in inhibition may have been due to the different aged leaf tissue which was used and the level of inhibitory substances, no doubt, changed with environmental conditions.

Boiled extracts of the two species prepared with and without PVP exhibited inhibitory properties (Table 21) similar to fresh extracts (Table 20). This indicated that the inhibitors were not proteinaceous and not heat labile. The inhibitor was also very stable with time as ericaceous extracts kept at room temperature for one week were completely inhibitory to active corn extracts.

The dialyzed ericaceous extracts prepared without PVP were completely inhibitory to corn nitrate reductase (Table 22) adding further evidence that the inhibitors were nondialyzable. Fresh and boiled dialyzed PVP prepared extracts of rhododendron were 50% inhibitory while leucothoe extracts (Table 22) were 30% inhibitory. This provided additional evidence that PVP was capable of removing a percentage of the inhibition and that leucothoe probably does not possess the high level of inhibitors as does rhododendron.

More conclusive information as to the precise nature of the inhibitors was obtained via the fractionation study. The fact that the resuspended ericaceous acetone pellets were not inhibitory (Table 23) indicated positively that protein was not responsible for inactivation. On the other hand, the resuspended concentrates were completely inhibitory while PVP treated resuspended concentrates were approximately 20% inhibitory. This in itself was strong evidence that the inhibitors were predominately phenolic in nature.

The spectrophotometric determination with the resuspended ericaceous concentrates provided stronger evidence that phenolics were the inhibitory substances. Leucothoe extracts (Figure 8) and rhododendron extracts (Figure 9) contained qualitatively twenty-fivefold more phenolics than extracts treated with saturating concentrations of PVP. The relationship between decreased inhibition and loss of phenolics by PVP treatment was strong evidence that phenolics were the inhibitory substances.

The fact that the simple phenolics are not effective protein inactivators while tannins (89) are potent irreversible protein inactivators indicated that the efforts to identify this heat stable, nondialyzable, phenolic type compound should be geared to a 'tannin-like' compound. Tannic acid was the only compound to affect nitrate reductase activity in corn (Table 24) while the simple phenolics had no effect. This suggested that the ericaceous extracts contained quantities of a 'tannin-like' substance which when released from the vacuole reacted with the viable protein and inactivated it. The resuspended acetone pellets did not give a positive test with biuret (28), which indicated the protein was probably lost in the first extraction step. Care was taken to insure that the 'protein' pellet was resolubilized by dissolving the pellets in both 0.1 M phosphate buffer, pH 7.5, and 0.1 M NaOH. In both instances no protein was detected.

Inhibition to nitrate reductase by tannic acid at 10^{-3} and 10^{-4} M was completely removed by treating the tannic acid solutions with saturating quantities of PVP indicating that tannic acid or a similar derivative were responsible for inhibition and that the reduction in inhibition by PVP treatment was due to the removal of 'tannin-like' compounds from the ericaceous extracts. All inhibition was not removed by PVP (Table 23) indicating that another molecular species was contributing a part of the inhibition.

Dialysis treatments were usually ineffective in freeing the system of inhibitors except in isolated cases where a small percentage of the original leucothoe inhibition was lost. Tannic acid was nondialyzable, for after 48 hours of dialysis tannic acid dialysates at 10^{-3} M were completely inhibitory to corn nitrate reductase. This provided further corroborative evidence that the nondialyzable inhibitors in ericaceous extracts were of a tannic acid nature.

Additional evidence for a tannic acid related inhibitor was provided by the chromatography work. Both leucothoe and rhododendron concentrated extracts had R_f values which corresponded to the R_f value of the commercial tannic acid and also gave positive colormetric tests indicating that a tannic acid type compound was the responsible agent. Even more conclusive was the fact that the eluate from the R_f value corresponding to the known tannic acid fraction inhibited nitrate reductase activity in corn. Inhibition from both ericaceous eluates was not complete but this may have been due to the small amount of material eluated from the chromatogram. Concentrated ericaceous extracts treated with saturating concentrations of PVP had no band of R_f value comparable to the known tannic acid fraction and also gave negative colormetric tests with $FeCl_3$ or $FeNH_4(SO_4)_2$.

The evidence then was very strong, although not conclusive, that a tannic acid-like compound was responsible for the bulk of the inhibition in both leucothoe and rhododendron extracts. All the data taken collectively indicated that the simple phenolics had little or no effect on enzyme inactivation and only when they are acted upon by the polyphenoloxidases (73) do they significantly affect enzyme extraction. Relatively pure solutions of chlorogenic, quinic, pyrogallol,

hydroquinone, rutin, juglone,and quercitin, had no effect on nitrate reductase activity in corn. Tannic acid was shown to be a powerful protein inactivator, this was further evidence that during extraction of the enzyme systems by the homogenate method, tannic acid-like compounds were responsible for failure to extract an active enzyme fraction.

Bacterial Study

There were no differences in nitrate reductase among the various treatments (Table 25), this indicated that bacteria were not influencing the observed rates of reduction from leucothoe leaves. If anything, bacteria were reducing the nitrite content of the system, for those leaf pieces which contained the greatest bacterial population, showed the lowest nitrate reductase activity (Table 25). The use of chloramphenicol was added protection against bacterial contamination. Chloramphenicol (79) is a potent inhibitor of protein synthesis in bacteria and therefore prevented bacterial growth while the leaf pieces were in the incubation medium. Chloramphenicol has been shown to have no effect on nitrate reductase induction, degradation, or activity and therefore was in no way influencing the rates of activity (79). Nitrate reductase in incubated leaves treated with and without chloramphenicol was essentially the same indicating again that bacteria were not affecting the observed nitrate reductase activity.

Mycorrhizal Study

The absence of mycorrhizae in greenhouse grown ericaceous roots (Figures 11 A, B, C and D) indicated that these fungi played no role in nitrate reduction. Actually, there is very little evidence concerning fungi and their ability to reduce NO_3-N (54). However, since fungi were not present in the roots, this supported the fact that the observed rates of reduction were due to the plant system.

The wild blueberry contained appreciable amounts of mycorrhizal infection. It has been well documented that the mycorrhizal infections of the Ericaceae are of an endotrophic nature (44). This means that the hyphae actually penetrate the host cell and live symbiotically. Proof of this is evident in Figure 12 D where the hyphal strands appear as pieces of string within the cell wall outline. Another interesting point was that root hairs were completely absent from uninfected roots (Figures 11 A, B, C and D) while what appears to be root hairs (Figures 12 A, B and C) were present in infected roots. Apparently, these root hair-like appendages are simply extensions of the fungi's hyphal system (44). However, it was also reported (52) that once roots were infected the epidermal cells of the ericaceous root developed normal root hairs. The development of these root hairs may be of benefit to the ericaceous plants which grow in poor, infertile soils as they facilitate the uptake of greater amounts of water and minerals.

SUMMARY

Studies were conducted to determine whether: (1) NO_3-N could serve as a suitable N source for woody ornamental ericaceous plant growth and development; (2) pH would affect growth; (3) the presence of a nitrification inhibitor would affect growth; (4) Ericaceae were capable of reducing NO_3-N; (5) the inhibitory substances were of a phenolic nature and; (6) bacteria and fungi affected the observed levels of nitrate reduction in the leaves and roots of the Ericaceae. The results obtained from this work indicated the following:

1) Nitrate N was superior to both urea and NH_4-N in promoting the growth of leucothoe and rhododendron when applied at 100 ppm daily.

2) Urea and NH_4-N were toxic at 100 ppm and therefore should not be used as N sources where continuous fertilization is employed.

3) The pH per se had little effect on growth and only through its effect on ion availability, microbial activity, and other factors does it exert an effect on growth.

4) The use of a nitrification inhibitor is unwarranted in view of the negative results obtained in these studies.

5) Nitrate reductase was present in all ericaceous species examined indicating that these plants can utilize NO_3-N for their growth and development.

6) The detection of activity was dependent on the extraction and assay technique employed.

7) The inhibitors which confounded the extraction of an active nitrate reductase fraction from ericaceous plants and also inhibited activity in corn extracts were tannic acid-like in nature.

8) Bacteria and fungi played no role in nitrate reduction indicating the observed rates of reduction in the leaves and roots of Ericaceae were due to the plant's reductase system.

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APPENDIX

TABLE 1

NUMBER OF SHOOTS OF LEUCOTHOE CATESBAEI

N Serve Level	N Source: Urea	NO_3-N	NH_4-N	$\bar{x}$
0 ppm	9.8 b [1/] w	13.4 a w	5.5 c w	9.6 w
40 ppm	15.1 a x	11.3 b w	6.8 c w	11.1 w
$\bar{x}$	12.5 a	12.4 a	6.2 b	

[1/] Means not followed by the same letter in sequence are significantly different at the 5% level.

TABLE 2

NUMBER OF SHOOTS OF LEUCOTHOE CATESBAEI

pH Level	0 ppm N Serve N Source Urea	NO_3-N	NH_4-N	$\bar{x}$	40 ppm N Serve N Source Urea	NO_3-N	NH_4-N	$\bar{x}$
4 to 5	11.5a[x/] w	12.7a w	4.0b w	9.4 w	14.0a w	13.8a w	5.8a w	11.2 w
6 to 7	8.0b w	14.0a w	7.0b w	9.7 w	16.3a w	8.8b x	7.8b w	11.0 w
$\bar{x}$	9.8b	13.4a	5.5c		15.2a	11.6c	6.9c	

[x/] Means not followed by the same letter in sequence are significantly different at the 5% level.

TABLE 3

LENGTH (IN.) OF SHOOTS OF LEUCOTHOE CATESBAEI

N Serve Level	N Source Urea	NO_3-N	NH_4-N	$\bar{x}$
0 ppm	54.6b[x/] w	71.5a w	26.3c w	50.8 w
40 ppm	66.9a w	47.8b x	26.0c w	46.9 w
$\bar{x}$	60.8a	59.8a	26.2b	

[x/] Means not followed by the same letter in sequence are significantly different at the 5% level.

TABLE 4

LENGTH (IN.) OF SHOOTS OF LEUCOTHOE CATESBAEI

pH Level	0 ppm N Serve N Source				40 ppm N Serve N Source			
	Urea	NO_3-N	NH_4-N	$\bar{x}$	Urea	NO_3-N	NH_4-N	$\bar{x}$
4 to 5	64.0a[x/] w	62.0a w	23.5b w	49.8a w	58.8a w	61.2a w	11.8b w	43.9 w
6 to 7	45.3b w	81.0a w	29.0b w	51.8 w	75.0a w	34.0b x	40.3b x	49.8 w
$\bar{x}$	54.7a	71.5a	26.3b		66.9a	47.6	26.1b	

[x/] Means not followed by the same letter in sequence are significantly different at the 5% level.

TABLE 5

NITRATE NITROGEN CONTENT OF YOUNG LEUCOTHOE CATESBAEI LEAVES

N Serve Level	N Source Urea	NO_3-N	NH_4-N	$\bar{x}$
0 ppm	1.32 a[x/] w	0.61 b w	0.82 b w	0.92 w
40 ppm	0.81 b x	0.58 b w	1.39 a x	0.93 w
$\bar{x}$	1.07 a	0.60 b	1.11 a	

[x/] Means not followed by the same letter in sequence are significantly different at the 5% level.

TABLE 6

NITRATE NITROGEN CONTENT OF OLD LEUCOTHOE CATESBAEI LEAVES

N Serve Level	N Source Urea	NO_3-N	NH_4-N	$\bar{x}$
0 ppm	1.66 a [x/] w	0.84 c w	1.31 b w	1.27 w
40 ppm	1.03 a x	0.60 b x	1.09 a x	0.91 x
$\bar{x}$	1.35 a	0.72 c	1.20 b	

[x/] Means not followed by the same letter in sequence are significantly different at the 5% level.